Non-autonomous regulation of bone mass accrual and the role of T-cell protein tyrosine phosphatase in the bone regulation of insulin sensitivity

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Submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
in the Graduate School of Arts and Sciences

COLUMBIA UNIVERSITY

2013

Abstract

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The skeleton is a highly dynamic organ that undergoes constant remodeling to renew itself and maintain bone mass. It is subject to regulation from both hormones produced in peripheral tissues and neuronal control by the nervous system. Recent studies have shown that the skeleton is also an endocrine organ, releasing the hormone osteocalcin that increases insulin secretion and sensitivity in the pancreas and testosterone production in the Leydig cells of the testis. In my thesis study, I explore both the cell-nonautonomous regulation of osteoblast differentiation and the bone regulation of energy metabolism using cell-specific gene inactivation in the mouse.

Early B-cell factor 1 (Ebf1) is a transcription factor whose inactivation in all cells results in high bone mass because of an increase in bone formation. To test if Ebf1 regulates bone formation cell-autonomously, I analyzed *Ebf1* pattern of expression and its function in osteoblasts. I show here that *in vivo* deletion of *Ebf1* in osteoblast progenitors does not affect osteoblast differentiation or bone formation accrual post-natally, indicating that the phenotype described in $Ebf1^{-/-}$ mice is not osteoblast-autonomous.

Insulin signaling in osteoblasts contributes to whole body glucose homeostasis in the mouse and in humans by increasing the activity of osteocalcin. The osteoblast insulin signaling cascade is negatively regulated by ESP, a tyrosine phosphatase dephosphorylating the insulin receptor. *Esp* is one of many tyrosine phosphatases expressed in osteoblasts, and this observation

suggests that other protein tyrosine phosphatases may contribute to the attenuation of insulin receptor phosphorylation in this cell type. In this study, we sought to identify additional PTP(s) that like ESP, would function in the osteoblast to regulate insulin signaling and thus affect activity of the insulin-sensitizing hormone osteocalcin. For that purpose, we used as criteria, expression in osteoblasts, regulation by isoproterenol, and ability to trap the insulin receptor in a substrate-trapping assay. Here we show that the T-cell protein tyrosine phosphatase (TC-PTP) regulates insulin receptor phosphorylation in the osteoblast, thus compromising bone resorption and bioactivity of osteocalcin. Accordingly, osteoblast-specific deletion of TC-PTP (*Ptpn2*) promotes insulin sensitivity in an osteocalcin-dependent manner. This study increases the number of genes involved in the bone regulation of glucose homeostasis.

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Chapter One: General Introduction

Bone development and physiology

A defining feature of higher order organisms is the presence of a bony skeleton. Totaling 206 bones, the human skeleton is composed of 80 axial bones and 126 bones appendicular bones. The bones are shaped during development, extended during growth, and regenerate continuously throughout adulthood. The skeleton fulfills multiple functions in the body. First, it provides a rigid frame to support the body's form. The architecture of the skeleton is such that it provides protection to vulnerable soft tissues like the heart and brain, but allows for a wide range of movement at the joints, which are formed like a ball-and-socket. Secondly, the skeleton is a store of minerals and growth factors that are confined to the bone extracellular matrix and released into circulation upon bone remodeling. Third, the bone marrow is the site of hematopoiesis. Fourth, it was shown recently that the skeleton is an endocrine organ, releasing at least two bone-specific hormones, FGF23 and osteocalcin, that affect other tissues of the body.

Formed during development, the skeleton undergoes constant postnatal remodeling throughout growth and adulthood. The skeleton is composed predominantly of three kinds of cells: the chondrocyte, which form the cartilage; the osteoblast, which are responsible for the structure and mineralization of the bone; and the osteoclast, which resorb the matrix for remodeling. Both chondrocytes and osteoblasts are of mesenchymal origin, while the osteoclasts differentiate from the hematopoietic lineage.

Our understanding of skeletal development and physiology has been greatly improved by studies performed in the mouse. As will be discussed below, many human skeletal genetic diseases parallel a corresponding knockout model in the mouse, suggesting that the genetic cascades in bone are well conserved between rodents and humans.

Development of the skeleton

The skeleton is formed in the embryo from condensing mesenchyme. Ossification of the mesenchyme occurs through two distinct processes: intramembranous and endochondral ossification. During intramembranous ossification, cells of the mesenchymal condensations directly differentiate into osteoblasts, which mature and produce mineralized tissue. This type of ossification forms the bones of the skull, mandibles, and clavicles – bones that are considered "flat."

In contrast, a process termed endochondral ossification forms "long" bones, such as the femur and tibia. In this 2-step process, condensed mesenchyme first differentiates into chondrocytes, which produce a cartilaginous extracellular matrix made up of type II collagen fibers, proteoglycans, glycoproteins, and hyluronan, and elastin fibers. Specification of the osteochondroprogenitor requires the transcription factors Runt-related transcription factor 2 (Runx2) and Sex-determining region Y box 9 (Sox9), both expressed at E10.5. Sox9, the activity of which is partially determined by Sox5 and 6 transcription factors, is the master determinant of chondrogenesis (Figure1-1) (Bi et al., 1999; Inada et al., 1999; Kim et al., 1999; Lefebvre et al., 2001; Lefebvre et al., 1997). It is directly responsible for the transcription of the proteoglycan gene *Aggrecan,* and the type II collagen gene *Col2α1* (Figure 1-1) (Bell et al., 1997; Ng et al., 1997). Mutations in the human *SOX9* gene lead to camptomelic dysplasia, a kind of skeletal dysplasia characterized by bowing of the long bones (Lefebvre et al., 1997).

The chondrocytes proliferate longitudinally along the bone, secreting extracellular matrix and pushing older chondrocytes towards the midsection of long bone. The older chondrocytes undergo hypertrophy and drastically increase their secretion of type X collagen and osteonectin into the extracellular matrix (Pacifici et al., 1990). Chondrocyte maturation is dependent upon

the both the transcription factors runt-related 2 (Runx2) and 3 (Runx3), members of the Runt DNA binding domain-containing transcription factor family and are partially redundant in chondrocytes (Figure 1-1) (Yoshida et al., 2004).

The second phase of endochondral ossification begins when vessels invade the region of hypertrophic chondrocytes. These chondrocytes then undergo apoptosis and are replaced by osteoblasts originating in the bone collar surrounding the cartilage template. Growth of long bones involves the extension of chondrocyte proliferation longitudinally to guide future ossification.

Osteoblasts and the bone extracellular matrix

The osteoblasts that ossify the bony skeleton are specialized cells that produce an extracellular matrix mainly made of type I collagen fibers that become mineralized. This extracellular matrix contains numerous non-collagenous proteins. Mineralization of the bone extracellular matrix involves deposition of $Ca_5(PO_4)_3(OH)$ hydroxyapatite within the fibrous scaffold. Mineralization gives bones their characteristic rigidity and resilience to compressive force.

Transcriptional regulation of osteoblast differentiation

Runx2, responsible for terminal differentiation of chondrocytes, is also expressed in osteoblasts where it is the master transcriptional regulator of osteoblast specification and differentiation. Its Runt DNA-binding domain is a clamp-like structure that encircles the DNA for a stable protein-DNA interaction. The Runt family of transcription factors also contains a conserved C-terminal proline-serine-theronine-rich (PST) domain and a nuclear localization

signal. However, Runx2 is unique from Runx1 and 3 in that it has three activation domains that control its transcription activation and prevent interaction with the Runx transcriptional partner Cbfβ (Thirunavukkarasu et al., 1998).

Runx2 expression is initiated in the sites of skeletal condensation at E10.5 and increases steadily in the sites of ossification until birth (Ducy et al., 1997). Post-natally, *Runx2* expression becomes restricted to cells of the osteoblast lineage, where it promotes bone formation by differentiated osteoblasts (Ducy et al., 1999). Mice lacking *Runx2* die at birth from severe skeletal dysplasia, lacking any ossification (Otto et al., 1997). In humans, missense mutations in *Runx2* are associated with cleidocranial dysplasia, a disorder characterized by collarbone and cranium deformities and short stature from underdeveloped bones and joints (Mundlos et al., 1997) (Lee et al., 1997).

As the master transcriptional regulator of osteoblastogenesis, Runx2 is regulated at several levels (Fig. 1-1). At the level of expression, Runx2 is positively regulated by muscle segment homeobox homolog 2 (Msx2) at the sites of endochondral ossification, and bagpipe homeobox gene (Bapx1) in the axial skeleton. Both *Msx2*$^{-/-}$ and *Bapx1*$^{-/-}$ mutant mice display deficiencies in ossification due to decreased expression of *Runx2* in the osteoblasts (Satokata et al., 2000; Tribioli and Lufkin, 1999). Humans with loss-of-function mutations *MSX2* develop enlarged parietal foramina (PFM), characterized by openings in the skull, while gain-of-function *MSX2* mutations result in Boston-type craniosynostosis, a disease marked by premature ossification and high bone density of craniofacial elements (Karsenty, 2008; Satokata et al., 2000; Wilkie et al., 2000). In contrast, Homeobox A2 (Hoxa2) negatively regulates *Runx2* expression, and *Hoxa2*$^{-/-}$ mice display ectopic bone formation in the second branchial arch as a result of increased *Runx2* expression (Kanzler et al., 1998).

The DNA binding domain of Runx2 is also a site of post-translational regulation. Though *Runx2* is expressed at E10.5, ossification is undetectable until E14.5. Twist-1 is a basic helix-loop-helix (bHLH) transcription factor containing a Twist box, an interaction domain. The Twist box interacts transiently with the Runx2 runt domain to attenuate its activity from the E10.5 to E14.5 window and thus inhibit osteoblast differentiation. At E14.5, *Twist-1* expression considerably decreases, permitting *Runx2*-dependent ossification to start (Bialek et al., 2004). Accordingly, in humans, a lack of *Twist-1* results in premature ossification, and mutations within *TWIST-1* are a major cause of craniosynostosis (el Ghouzzi et al., 1997). Stat1 (signal transducer and activator of transcription 1) binds to the Runx2 runt-domain, where it inhibits Runx2 nuclear translocation. Due to increased Runx2 transcriptional activity, *Stat1*$^{-/-}$ mice develop high bone mass (Kim et al., 2003a). A zinc-finger adaptor protein, Schnurri-3, regulates Runx2 degradation by recruiting E3 ubiquitin ligase WWP1 to Runx2 (Jones et al., 2006).

In contrast, Satb2 (Special AT-rich sequence-binding protein 2), a nuclear matrix protein, promotes Runx2 activity in two ways: it interacts directly with Runx2 to enhance its DNA binding and transactivation ability, and secondly, represses expression of the Runx2-inhibitor Hoxa2. Humans with a deficiency in *SATB2* thus develop abnormal craniofacial patterning (Dobreva et al., 2006).

Downstream of Runx2 is Osterix, a zinc-finger containing transcription factor specific to osteoblasts and chondrocytes that is necessary for further osteoblast differentiation and mineral deposition (Nakashima et al., 2002). Like Runx2, it is required both during development and post-natal growth for new bone formation during growth and remodeling (Zhou et al., 2010). Osterix activity is, in part, determined by its interaction with the transcription factors, nuclear factor of activated T-cells NFATc1 and c2 (Koga et al., 2005).

Terminal osteoblast differentiation, defined by the ability of the osteoblast to mineralize the extracellular matrix, is dependent upon ATF4 (activating transcription factor 4), a member of the family of the ATF-CREB bZIP family of transcription factors that contains a basic leucine-zipper. ATF4 stimulates the synthesis of type I collagen by promoting amino acid import into the osteoblasts (Harding et al., 2003). In addition, ATF4 regulates *Rankl* expression to control osteoclast differentiation, and thus bone resorption, as discussed in a subsequent section (Elefteriou et al., 2006).

Molecular regulation of ATF4 activity occurs at the post-translational level. The leucine zipper domain of ATF4 is subject to phosphorylation. RSK2, a growth factor-related kinase, phosphorylates ATF4 to promote its transcriptional activity. Loss-of-function mutations in *RSK2* result in Coffin-Lowry syndrome, a multi-system disorder that also affects bone growth. Individuals with Coffin-Lowry, in addition to developmental delays and cardiac abnormalities, have brittle bones and short stature as a result of decreased ATF4 activity (Yang et al., 2004). ATF4 activity, like that of Runx2, is also enhanced by the binding of Satb2 (Dobreva et al., 2006). In contrast, Nf1 (Neurofibromatosis 1), a Ras-GTPase activating protein, is a negative regulator of Atf4 activity. It is an inhibitor of Ras/MAPK/Rsk2 signaling, of which ATF4 is downstream. Deletion of Nf1 specifically in osteoblasts results in increased collagen synthesis (Elefteriou et al., 2006).

Ebf1 and its regulation of bone mass accrual

An interesting candidate for and additional level of regulation of osteoblast differentiation is the transcription factor Early B-cell factor 1 (Ebf1). Ebf1 belongs to the Ebf/COE family of transcription factors. Members of this family share a highly conserved

DNA-binding domain, a transcription factor immunoglobulin (TIG/IPT) domain, and an atypical Helix-Loop-Helix (HLH) motif. The DNA binding domain contains an unusual zinc-finger like motif, designated the "zinc-knuckle," that is essential for its transcriptional activity (Figure 1-2). Ebf1 binds to DNA as a symmetric dimer in a stable configuration that maximizes protein-DNA contact at 3000Å^2. The observation that Ebf1 has one of the largest known protein-DNA interfaces led to the hypothesis that Ebf1 may be a "pioneer" transcription factor that acts early in cell specification to stabilize chromatin accessibility for downstream factors (Hagman et al., 1995; Treiber et al., 2010a).

There are four *Ebf* paralogs in vertebrates (Ebf2, 3, and 4). Ebf1, working in concert with E2A and Pax5, is a principle determinant of B-cell fate specification and commitment and expressed in all stages of B cell differentiation. Common lymphoid progenitors lacking *Ebf1* disproportionately differentiate into myeloid and T cell lineages at the expense of mature B cells. Specifically, *Ebf1*$^{-/-}$ cells arrest at the pre-pro-B stage (Lin and Grosschedl, 1995; Treiber et al., 2010b). By comparison *Pax5*$^{-/-}$ (*Paired box 5*), the major transcriptional guardian of B cell identity, arrest at the pro-B stage (Urbanek et al., 1994) (Nutt et al., 1997) (Cobaleda et al., 2007). Overexpression of *Ebf1* promotes B cell development at the expense of these lineages. Ebf1 acts to regulate expression of its target genes involved in pre-B cell receptor and Akt signaling, cell adhesion, and migration. Ebf1 activated targets are H3K4 methylated or H3 acetylated, while its repressed targets demonstrate loss of activating chromatin marks and a gain of H3K27 trimethylation (Treiber et al., 2010b).

Runx1 has recently been identified as an important transcription factor regulating Ebf1 expression and activity. Runx1 and Ebf1 regulate early B cell lineage specification genes and Ig gene rearrangement synergistically (Lukin et al., 2010). The activity of Ebf1 is in part

determined by Runx1, which binds to the *Ebf1* promoter region to drive its expression (Seo et al., 2012).

Ebf1 shares approximately 90% identity with its paralogues Ebf2 and 3. All three members are expressed in the olfactory epithelium, spinal cord and hindbrain regions. *Ebf1*$^{-/-}$ mice have normal olfactory neurons, and it is believed that its redundancy with Ebf2 and 3 may account for the lack of an olfactory neuron phenotype in *Ebf1*$^{-/-}$ mice (Wang et al., 1997). In addition to its expression in olfactory sensory neurons, *Ebf1* is present in the embryonic striatum where no other *Ebf* gene is expressed. It is important for mantle cell differentiation and migration in neuroepithelial progenitors (Garel et al., 1999), as well as striatonigral medium spiny neurons in the striatum (Lobo et al., 2008).

More importantly for our purposes, previous studies have implicated additional function for Ebf1in the mesenchymal lineage, specifically in adipocytes and osteoblasts. It is important to stress however, that the Ebf1 putative role in adipogenesis has been studied exclusively in cell culture through the use of the cell line 3T3L1 (Akerblad et al., 2002; Jimenez et al., 2007). These cells normally adopt a fibroblast-like morphology, but under certain cell culture conditions, 3T3L1 cells express peroxisome proliferator-activated receptor γ2, the master transcriptional regulator of adipogenesis. Continued "differentiation" of these cells results in the production of trigylcerides and lipid droplets. The conditions that promote 3T3L1 adipocyte-like differentiation include growth in medium containing either a PPARγ agonist (for example, Rosiglitazone), or a cocktail of pro-adipogenic agents: dexamethasone (a steroid), insulin, and IBMX (an immunosuppressant that raises cAMP levels) termed DMI (Green and Kehinde, 1975; Gregoire et al., 1998).

Jimenez et al. demonstrated that DMI differentiation induces an increase in the expression of Ebf1 and Ebf2 prior to the induction of PPARγ2 and CCAAT/enhancer-binding protein alpha (C/EBPα), a bZIP transcription factor part of a feed-forward loop that increases PPARγ2 activity and promotes adipocyte-specific gene expression (Jimenez et al., 2007). 3T3L1 cells lacking *Ebf1* have impaired adipogenesis in DMI medium, while *Ebf1* overexpression increases the formation of lipid droplets (Akerblad et al., 2002). These observations led to the hypothesis that Ebf1 may be a pre-PPARγ2 transcriptional determinant of adipogenesis.

Ebf1$^{-/-}$ mice are dwarfed and demonstrate increased bone mass and diminished subcutaneous and abdominal white adipose depots. The observations from cell culture experiments together with the lack of white fat depots in *Ebf1*$^{-/-}$ mice suggest that Ebf1 promotes adipogenesis. Since both adipocytes and osteoblasts are derived from mesenchymal cells, it has been hypothesized that Ebf1 could be an early mesenchymal transcriptional determinant of the adipocyte versus osteoblast cell fate decision (Fretz et al., 2010; Hesslein et al., 2009).

The metabolic profile of *Ebf1*$^{-/-}$ mice is expectedly altered. Previous analysis shows that these mice are chronically hypoglycemic with increased insulin sensitivity. They display increased energy expenditure and food intake, despite a slightly cachectic appearance. Due to increased bone mass and decreased adipose, serum osteocalcin and leptin levels are increased and decreased, respectively, in these mice. Interestingly, *Ebf1*$^{-/-}$ mice display an increase in marrow adipose, unusual given the fact that their white adipose depots are decreased (Fretz et al., 2010).

Bone resorption

Antagonistic to the bone-building properties of osteoblasts, osteoclasts resorb the organic and inorganic components from the bone matrix. The balance between the two opposing forces is integral to the constant process of bone regeneration, termed remodeling. A defect in resorption results in osteopetrosis, a condition characterized by hardening of the bone due to the imbalance toward bone formation. This condition also disrupts the brittleness of mineralized bone trabeculae, affecting hematopoiesis due to constriction of the bone marrow. In contrast, an increase in osteoclast function results in the condition osteoporosis. This disease, common especially in post-menopausal women, is marked by decreased bone mass and an increased risk of fractures.

Osteoclast structure and function

The osteoclast is a large, multinucleated cell formed from the fusion of 15-20 differentiated macrophages (Figure 1-3) (Boyle et al., 2003; Walker, 1975). To resorb the mineralized bone matrix, the osteoclast attaches its ends to the bone matrix using specialized attachment molecular and focal adhesions to form the sealing zone. Lysosomal vesicles are then exocytosed towards the bone surface, forming a cavity between the osteoclast and bone surface. The plasma membrane that forms the transportation border folds upon itself to facilitate the high rate of exocytosis, adopting a "ruffled" appearance. The process of establishing an osteoclast resorption lacuna requires polarization of the osteoclast, and the cytoskeleton accommodates these changes by reorganizing its cytoskeleton. C-src, a tyrosine kinase, is important for the phosphorylation and reorganization of integrins (such as αvβ3) and focal adhesions. A lack of *c-src* results in severe osteoclast dysfunction and osteopetrosis (Soriano et al., 1991)

The first step of bone resorption is dissolution of the inorganic matrix, achieved by acidification of the resorptive lacunae (Baron et al., 1985). Protons are generated by carbonic anhydrase II (CAII) and then transported to the lacunae by Tcirg1, a vacuolar proton ATPase expressed on the ruffled membrane (Blair et al., 1989; Kenny, 1985; Raisz et al., 1988). The acid microenvironment created by osteoclasts dissolves hydroxyapatite, releasing calcium and phosphate ions into the lacunae. These ions are absorbed by the osteoclast via endocytosis and transported across the cell and then released into the blood. Mutations in the human *TCIRG1* gene account for approximately half of human malignant infantile osteopetrosis cases (Frattini et al., 2000; Susani et al., 2004)

Maintenance of electroneutrality in the lacunae and osteoclast is also important for resorption. Chloride channel 7 (CLCN-7), localized to the ruffled membrane, transports chloride anions to the lacunae to balance the influx of protons into the lacunae. This protein is responsible for bringing the microenvironment pH to ~4.5 (Schlesinger et al., 1997). Mutations in *CLCN-7* disrupt the acidification of the lacunae, and result in osteopetrosis by definition (Kornak et al., 2001). A chloride/bicarbonate exchanger, localized on the membrane facing the extracellular fluid, corrects the osteoclast's internal proton efflux (Teti et al., 1989).

This process of bone resorption exposes the organic matrix, largely composed of type I collagen. Cathepsin K (Ctsk) is a collagenolytic protease that is released into the resorption lacunae to degrade the demineralized collagen matrix. It is processed in the Golgi and transported to the ruffled membrane, where it is secreted into the lacunae through exocytosis. A lysosomal protease, Ctsk is active in the acid pH of the lacunae, where it completes the resorption process by catabolizing the exposed collagen matrix (Bossard et al., 1996; Yamaza et al., 1998). Mice lacking the *Ctsk* gene possess fully differentiated osteoclasts, but develop

osteopetrosis due to the inability of the osteoclast to remove demineralized bone (Saftig et al., 1998). Pycnodysostosis, an autosomal recessive form of osteopetrosis characterized by short stature, craniofacial abnormalities, and defective bone resorption, is caused by a mutation in *CTSK (Gelb et al., 1996)*.

Mechanisms regulating osteoclast differentiation

Osteoclasts are of hematopoietic monocyte/macrophage lineage, formed in the bone marrow by the fusion of differentiated macrophages. A combination of transcription factors and extracellular signals are necessary for monocytes to fuse and differentiate into multinucleated, resorptive osteoclasts (Figure 1-4).

Transcriptional Control of Osteoclast differentiation

Unsurprisingly, the transcription factors necessary for monocyte differentiation are also essential for early osteoclastogenesis. PU.1, a transcription factor specific to hematopoietic cells, is the earliest known transcription factor known for early macrophage determination. Lack of a functional *PU.1* gene, characterized by an absence of macrophages and osteoclasts, is lethal (Tondravi et al., 1997).

Downstream of PU.1 are the microphythalmia-associated family of transcription factors (MITF), which include TFE3, TFEB, TFEC, and MITF (Weilbaecher et al., 2001). These helix-loop-helix transcription factors help to specify the differentiated macrophage/osteoclast precursor, and in later stages, interact with PU.1 to increase expression of osteoclast genes like *Ctsk* and *Trap* (*Tartrate-resistant acid phosphatase*), a histological marker of mature osteoclast function (Luchin et al., 2001).

The AP-1 transcription complex, which is downstream of JNK MAPK signaling, includes c-Fos, c-Jun, and JunB, and Fra/Fosl1. c-Fos itself is necessary for expression of the AP-1 transcriptional component *Fra1/Fosl1*. Though it is still unclear what are the targets of the AP-1 complex, it is known that its activity is required for osteoclastogenesis. Mice lacking genes involved in the AP-1 complex ($Fos^{-/-}$ and $Fosl1^{-/-}$) develop a sufficient number of macrophages, but lack mature osteoclasts (Wagner and Matsuo, 2003) (Matsuo et al., 2000) (Teitelbaum and Ross, 2003)

After osteoclast cell fate commitment, the osteoclast precursors fuse to form a multinucleated cell. The master regulator of osteoclast cell fusion and maturation is the nuclear factor of activated T-cells c1 (NFATc1). It is downstream of NF-κB signaling, and cooperates with c-Fos synergistically on the promoter of many functional resorptive genes, such as *TRAP*, *CAII*, and *Ctsk* (Takayanagi et al., 2002). Deletion of *Nfatc1* in the mouse results in a form of osteopetrosis lacking mature osteoclasts (Aliprantis et al., 2008).

Paracrine factors affecting osteoclastogenesis

Differentiation, proliferation, and survival of the osteoclast precursors are dependent on the paracrine factor M-CSF (macrophage colony-stimulating factor). M-CSF is secreted from nearby osteoblasts and binds to its receptor on immature macrophages, promoting MITF activity in a MAPK-dependent manner (Weilbaecher et al., 2001). In addition, M-CSF stimulates the ERK1/2 and PI3K/Akt signaling cascades to promote survival and proliferation of the immature macrophages (Gingery et al., 2003). Lack of M-CSF (*op/op*) in the mouse results in osteoclast deficient osteopetrosis (Kodama et al., 1991).

A second paracrine factor and critical determinant of osteoclast maturation is receptor activator of nuclear factor kappa B ligand (RANKL) signaling. RANKL, a member of the tumor

necrosis factor family, is expressed on the surface osteoblasts/stromal cells where it binds to the receptor RANK on neighboring osteoclast precursors (Lacey et al., 1998; Yasuda et al., 1998b). RANK activation recruits TNF receptor associated factor 6 (TRAF6), an adaptor protein that, in cooperation with c-Src, activates Akt/PI3K signaling pathway to initiate cellular reorganization of integrins. In addition, RANKL-recruited TRAF6 initiates the NF-κB pathway and all three MAPK pathways to promote the activity of osteoclast differentiation transcription factors (Bai et al., 2008; Lamothe et al., 2007).

The RANKL/RANKL/OPG system

RANKL is the most powerful activator of osteoclastogenesis. It was first discovered as a factor that was both necessary and sufficient for the *ex vivo* differentiation of bone marrow-derived osteoclast precursor cells into mature osteoblasts. (Lacey et al., 1998; Udagawa et al., 1990). Accordingly, mutations in the *RANKL* gene result in osteopetrosis in both mouse and humans (Kong et al., 1999; Sobacchi et al., 2007).

Though osteoblasts are a major source of RANKL for the activation of osteoclast maturation, they are also a negative regulator of osteoclast differentiation. Osteoprotegrin (OPG) is a decoy receptor belonging to the TNF receptor family that is secreted by osteoblasts/stromal cells. By mimicking RANK receptor structure, OPG competitively hinders RANKL binding to its receptor on osteoclast precursors (Figure 1-5) (Simonet et al., 1997; Takahashi et al., 1988; Takai et al., 1998; Yasuda et al., 1998a). A shift in the equilibrium between osteoblast RANKL and OPG expression, the ratio of which is often used as an index for osteoclastogenesis, can thus affect osteoclast differentiation and resorptive activity. One such example is parathyroid hormone (PTH), which enhances bone resorption. PTH binds to its receptor on the osteoblast where it simultaneously increases the expression of *RANKL* while inhibiting that of *OPG* (Huang

et al., 2004). In contrast, estrogens have been shown to enhance *OPG* expression in osteoblasts, an observation suggestive of its role in the attenuation of bone resorption (Hofbauer et al., 1999).

An osteoblast transcriptional determinant of *Rankl* expression is ATF4. ATF4-activated *Rankl* expression is promoted specifically by Protein kinase A (PKA) signaling in the osteoblast. The osteoporotic phenotype observed in Neurofibromatosis type I (and phenocopied by $Nf1_{osb}^{-/-}$ mice) is accounted for by the increase in PKA signaling and increased Atf4-activated *Rankl* expression (Elefteriou et al., 2006).

Bone is an endocrine organ

A major advance in skeletal biology and in endocrinology has been the recent realization that the skeleton is an endocrine organ. One example of these is FGF23, which is released into circulation from osteoblasts and part of a feedback loop regulating phosphate homeostasis (Kawata et al., 2007; White et al., 2000). The other hormone is osteocalcin, a small protein secreted specifically by the osteoblasts and stored in the bone matrix. Upon bone resorption, osteocalcin is decarboxylated, i.e. activated, in the resorptive lacunae and released into the circulation where it promotes insulin secretion, insulin sensitivity, energy expenditure, and testosterone production (Ferron et al., 2010a; Lee et al., 2007; Oury et al., 2011).

FGF23

The cell's need for phosphate is extensive. Phosphate is required for DNA and RNA, energy metabolism, as a second messenger in signal transduction, and for the modification of proteins and lipids. It is also needed for bone mineralization, and therefore not surprising that the majority of the body's phosphate is stored in the mineralized bone extracellular matrix and

released by bone resorption as necessary. A lack of phosphate in the body leads to the softening of bones and increase risk for fractures that defines rickets in children, or osteomalacia in adults (Jonsson et al., 2003; Kawata et al., 2007; Yamazaki et al., 2002).

FGF23 is a hormone that normalizes phosphate levels in the body. Increased amounts of phosphate levels in the serum are detected by the osteoblast and osteocytes, stimulating the production of FGF23, which is then secreted into circulation. FGF23 binds to a FGF1 receptor/Klotho complex in the kidney where it inhibits expression of the tubular renal phosphate transporters, NPT2a and NPT2c, in the kidney (Gattineni et al., 2009; Urakawa et al., 2006). As a consequence of FGF23 action in kidney, phosphate reabsorption in the proximal tubule is decreased while urinary phosphate excretion is increased.

Hypophosphatemic rickets/osteomalacia is characterized by a mutation that leads to the overproduction or increased stability of FGF23. In autosomal dominant rickets, a mutation in the *Fgf23* gene protects the protein from posttranslational cleavage (White et al., 2001) (Shimada, Endocrinology 2002). X-linked hypophosphatemia (XLH) is caused by mutations in the protease PHEX that also prevents FGF23 cleavage(Campos et al., 2003). The last example of FGF23-dependent osteomalacia is tumor-induced osteomalacia, where FGF23 is overproduced as a consequence of osteocarcinoma (Shimada et al., 2001).

Osteocalcin

Osteocalcin, or Bone-GLA protein, is a 5.8 kDa protein specifically secreted into circulation by osteoblasts and used for decades as a biochemical marker of bone formation. Recent studies from our lab have shown that osteocalcin is in fact a hormone that, once

decarboxylated, stimulates insulin production, insulin sensitivity, energy expenditure, and testosterone production(Lee et al., 2007; Oury et al., 2011).

Osteocalcin is produced in the osteoblast where it undergoes posttranslational processing. In the endoplasmic reticulum, vitamin-K dependent protein carboxylase (VKD) modifies the three glutamic acid residues of osteocalcin into γ-carboxyglutamic acid (Gla) residues, a process requiring both vitamin K and carbon dioxide (Hauschka et al., 1989a; Lian and Friedman, 1978). Carboxylated osteocalcin is then secreted into the bone matrix, where it has a high affinity for hydroxyapatite (Hauschka et al., 1989a)

Through the resorptive activity of osteoclasts, osteocalcin is released into circulation. As it passes through the acidic environment of the resorption lacunae, the post-translational carboxylation of osteocalcin is partially reversed. The undercarboxylated form of osteocalcin, where Glu residue 13 is uncarboxylated, is an active circulating hormone (Ferron et al., 2010a).

Osteocalcin promotes insulin secretion and sensitivity

Circulating, GLU13-undercarboxylated osteocalcin increases insulin secretion in the β-islet cells of the pancreas in two ways: by increasing β-islet cells proliferation, and by stimulating insulin production in the β-islet cells. Coculture experiments demonstrate that pancreatic islets increase their expression of the *Insulin* genes and of the proliferation genes *CyclinD1* and *CyclinD2* in the presence of primary osteoblasts, an effect that is dependent upon *Osteocalcin* expression (Lee et al., 2007).

In vivo, mice lacking osteocalcin ($Ocn^{-/-}$) display hyperglycemia and hypoinsulinemia. The pancreases of these mice have decreased β-islet size and number, insulin content, and proliferation. As a result, mice lacking osteocalcin are glucose intolerant and resistant to glucose stimulated insulin secretion (Lee et al., 2007).

In various human studies, total and undercarboxylated osteocalcin levels have been correlated with lower glucose and increased insulin levels, suggesting that the role of osteocalcin in the regulation of glucose homeostasis is conserved in humans (Im et al., 2008) (Kindblom et al., 2009) (Fernandez-Real et al., 2009) (Winhofer et al., 2010) (Hwang et al., 2009) (Shea et al., 2009). In addition, osteopetrotic patients display a decrease in undercarboxylated osteocalcin levels accompanied by an increase in insulin levels, suggesting that the osteocalcin activation process is also conserved (Ferron et al., 2010a).

Osteocalcin promotes testosterone production and male fertility

In addition to its role in promoting glucose homeostasis, osteocalcin stimulates testosterone production in Leydig cells of the testes. In mice lacking *Osteocalcin*, testis size was significantly lower with a corresponding decrease in testosterone production in the blood. Osteocalcin, released by the bone, binds to its receptor Gprc6a on the surface of Leydig cells. Upon binding of osteocalcin to Gprc6a, CREB is phosphorylated by PKA, and it then activates multiple genes that are involved in the synthesis of testosterone – *StAR*, *Cyp11*, *Cyp17*, and *3β-HSD*. Increased testosterone production in these mice directly resulted in increased sperm production (Oury et al., 2011).

One human association study performed in Japanese male diabetic patients demonstrated that increased undercarboxylated osteocalcin levels were correlated with free testosterone in the serum (Kanazawa et al., 2012). In addition, osteocalcin was correlated with increased testosterone levels during post-pubertal male growth (Kirmani et al., 2011).

Insulin promotes osteocalcin activation

Upon the discovery that osteocalcin stimulates insulin production, it became a question whether insulin would, in turn, reciprocate regulation upon osteocalcin activity in a feed-forward or feed-back mechanism (Karsenty and Oury, 2012).

Insulin signaling

The functional insulin receptor (InsR) is a transmembrane tyrosine kinase receptor (RTK) homodimer formed by α and β subunits connected by a disulfide bond. The β subunit spans the cell membrane and contains the tyrosine kinase on it cytosolic face, while the external α subunit is exposed to the cell surface where it physically binds to its ligand insulin. Once insulin binds to its receptor, InsR becomes phosphorylated, and then autophosphorylated on three tyrosine residues. This results in a structural reconfiguration and internalization of the receptor into a vesicle that enables recruitment of specific downstream effectors and subsequent phosphate transfer (Schlessinger, 2000).

There are many intracellular substrates of the insulin receptor, including the insulin-receptor substrates (IRSs), Src-homology-2-containing proteins (SHC), Cas-Br-M ectropic retroviral transforming sequence homologe (Cbl), Grb2-associated binder-1 (Gab-1) and STAT proteins (Figure 1-6). They become tyrosine phosphorylated upon binding to the insulin receptor, and thus become a "docking site" for downstream adaptor proteins. (Taniguchi et al., 2006).

Two main signaling cascades, the PI3K/AKT/PKB (PKB) and RAS-mitogen-activated protein kinase pathway (MAPK) pathways, are involved in insulin signaling. These pathways

contain many components, some of which cooperate and converge to promote glucose uptake from the cell and thus affect cell growth, proliferation, and differentiation

Activated by phosphorylated IRS-1, Phosphatidylinositol 3-kinase (PI3K) catalyzes the formation of the lipid second messenger PIP_3 from PIP_2 and ATP. This leads to recruitment of several PIP_3-dependent serine/threonine kinases (PDK) and AKT/PKB to the plasma membrane, where PDKs activates AKT/PKB. AKT/PKB targets: glycogen synthase kinase (GSK) to decrease glycogen synthesis; AKT substrate (AS160) to increase glucose transporter localization to the plasma membrane; mTOR to affect protein synthesis; and importantly, phosphorylates the Forkhead box protein O1 (FOXO1) transcription factor. The posttranslational modification of FOXO1 prevents it from entering the nucleus, and allows the expression of gluconeogenic and lipogenic enzymes.

Insulin signaling in the osteoblast

Deletion of the insulin receptor in osteoblasts ($InsR_{osb}^{-/-}$) results in increased adiposity, glucose intolerance, and insulin resistance in mice on a normal chow diet. This is in contrast to the deletion of the insulin receptor in skeletal muscle and white adipose tissue – mice do not display either phenotype unless they are challenged by a high fat diet, suggesting that bone is a significant contributor to whole body glucose homeostasis. This occurs because insulin signaling in osteoblasts promotes osteocalcin activation. Insulin signaling in the osteoblast affects the osteoblast production of osteocalcin in two ways: by promoting bone resorption and stimulating osteoblast development (Ferron et al., 2010a; Fulzele et al., 2010).

Opg expression is decreased upon the activation of the insulin receptor. The insulin signaling effectors Akt and FOXO1 in other cell types are also effectors in the osteoblast.

Phosphorylation of Akt, and subsequently FOXO1, results in the inhibition of *Opg* expression and increase in osteoclast function. The resorption lacunae that is necessary for osteocalcin release and activation is thus formed (Figure 1-7). Deletion of FOXO1 specifically in osteoblasts results in a gain of osteocalcin bioactivity, thus affecting whole-body glucose homeostasis (Ferron et al., 2010a).

As it does in other insulin-responsive cells, the insulin receptor affects osteoblast proliferation, survival, and differentiation. By increasing *Twist-1* inhibition of Runx2, a direct transcriptional regulator of *Osteocalcin*, insulin increases osteoblast proliferation and the production of osteocalcin, at the total and undercarboxylated level. Mice lacking *InsR* specifically in the osteoblasts display decreased osteoblast and bone formation rate, and as a result have decreased serum osteocalcin levels (Fulzele et al., 2010). FOXO1 may also be a mediator of the insulin control of osteocalcin production, as it is a major transcriptional regulator of osteoblast proliferation and acts in concert with ATF4 in the nucleus to control amino acid import and collagen synthesis (Rached et al., 2010b).

Regulation of insulin signaling by protein tyrosine phosphatases

Insulin resistance is a condition where cells fail to respond adequately to insulin and thus do not efficiently uptake glucose, amino acids, and/or lipids, resulting in their accumulation in the blood. If the pancreas is unable to produce enough insulin to clear the accrued glucose and free fatty acids, this condition develops into type 2 diabetes. Thus, it became particularly important to identify factors that attenuate the insulin signal. Protein tyrosine phosphatases (PTPs) are a family of enzymes that remove phosphate groups from phosphorylated tyrosine residues. They have since been identified as essential regulators of both insulin receptor and

insulin receptor substrate phosphorylation, the first "node" of insulin action (Taniguchi et al., 2006).

Protein tyrosine phosphatase catalytic mechanism

Protein-tyrosine phosphatases (PTPs) are defined by a conserved signature motif [I/V]HCXXGXXR[S/T], termed the "WPD loop," containing an essential cysteine in the catalytic site, the thiolate ion of which acts as a nucleophile to dephosphorylate its substrate (Tonks, FEBS Letters 2003). Catalysis begins first from nucleophilic attack by the thiolate ion upon the substrate phosphate group, thus binding the PTP to its substrate (Figure 1-8). This results in an 8-12 Å conformational change where the aspartic acid residue is brought close to the phosphoryl-cysteine intermediate. The aspartic acid residue then protonates the substrate tyrosyl-leaving group, thus releasing the substrate. In the second step, the deprotonated aspartic acid residue acts as a base to hydrolyze the phosphoryl-cysteine intermediate and release the phosphate group. This last step regenerates PTP activity (Figure 1-7) (Tonks, 2003).

Ligand-induced receptor tyrosine kinase (RTK) phosphorylation may be dependent upon the inactivation of PTPs. The observation that PTP inhibitors increase RTK autophosphorylation suggests that functional RTK dimers exist in the absence of ligand binding and that PTP activity prevents RTK phosphorylation (Schlessinger, 2000). One proposed model suggests that ligand-RTK binding induces activation of an NADPH oxidase, resulting in the generation of reactive oxygen species (ROS). The presence of ROS reversibly oxidizes the vulnerable thiolate ion of the PTP catalytic cysteine, converting it to sulfenic acid and then sulfenyl amide, preventing substrate interaction. By inactivating the PTP, RTK phosphorylation is permitted. PTP activity is restored upon exposure of the sulfenyl amide ion to glutathione or thioredoxin in the

intracellular fluid, ready to bind dephosphorylate its next substrate (Figure 1-9) (Meng et al., 2000; Meng et al., 2002; Salmeen et al., 2003; Tonks, 2005; van Montfort et al., 2003)

There are three classes of PTPs: classical, which dephosphorylate tyrosine resides; dual specificity, which dephosphorylate both serines/threonine and tyrosine residues; and pseudophosphatases, which lack a functional catalytic site but can affect RTK signaling in alternate ways. The classical PTPs are further divided into subgroups: receptor and non-receptor, or cytoplasmic, PTPs. Twelve of the receptor PTPs contain a second pseudo-phosphatase domain (D2) that has been shown to be inactive but necessary for the specificity and stability of the protein (Tonks, 2005). However, in the case of PTPRA, the D2 domain displays low residual activity (Krueger et al., 1990).

Dual-specificity PTPs are structurally similar to classical PTPs, but their active site accommodates phosphorylated serine and threonine residues. Preferential phosphorylation of the serine/threonine instead of the tyrosine residue, or vice versa, has been demonstrated for some of phosphatases in this class (Poon and Hunter, 1995; Schumacher et al., 2002).

Pseudophosphatases are those whose phosphatase domains are catalytically inactive. Originally believed to be nonfunctional remnants of evolution, deletions of particular pseudophosphatases have been shown to have disease-related consequences (Azzedine et al., 2003). Furthermore, these proteins maintain their ability to interact with other PTPs in a dominant-negative manner (Kim et al., 2003b; Tonks, 2005).

PTP substrate-trapping mutants

A useful biochemical tool in investigating the targets of PTPs is the use of substrate-trapping mutants. PTPs can be mutated into two types of substrate-trapping mutants, C/S and D/A. C/S mutants alter the catalytic cysteine to a serine, thereby forming a stable PTP–Ser–

PO_3–S complex. This substrate mutant permits binding (similar K_m) but blocks catalysis (decreases K_{cat}). However, this kind of mutation leaves the PTP resistant to regulation by reversible oxidation. In D/S mutants, the aspartate residue 181, the catalytic quench, is mutated to an alanine residue, allowing the PTP to bind to its physiological substrate in the cell, but unable to dephosphorylate it and release the substrate. In contrast to the C/S substrate trapping mutant, the D/A mutant retains the sensitivity of the catalytic cysteine to oxidation. The K_{cat} of the D/A mutant is severely decreased without substantially affecting the ability of the enzyme to bind to its substrate (K_m) (Blanchetot et al., 2005; Flint et al., 1997a).

ESP

ESP, or osteotesticular protein tyrosine phosphatase (OST-PTP), belongs to the class of classical receptor protein tyrosine phosphatases. It contains two phosphatase domains with a catalytically inert second domain (CD2). Expressed predominantly in the osteoblasts and Sertoli cells of the testis, ESP was postulated to be involved in osteoblast function for three reasons: its expression is upregulated by parathyroid hormone, *ex vivo* differentiation of rodent calvaria-derived osteoblasts increases its expression, and targeted knockdown of ESP results in abrogation of primary osteoblast differentiation (Mauro et al., 1996; Mauro et al., 1994) (Chengalvala et al., 2001; Dacquin et al., 2004).

In vivo during embryonic skeletogenesis, ESP expression is regulated temporally and spatially. At E12.5 its expression is detectable in the mesenchyme regions of cartilage and the craniofacial bones, ribs, limbs. From E14.5 to birth, expression becomes more localized to the bone collar of skeletal elements undergoing endochondral ossification and the skull regions of intramembranous ossification. At this point in development, ESP expression is excluded from regions of developing cartilage (Yunker et al., 2004). In the bone collar of long bone, the

expression of ESP colocalizes with osteoblast markers *Runx2* and *Col1a1*. In adult mice, ESP expression remains in the bone collar (Dacquin et al., 2004).

The expression of ESP in differentiating primary osteoblasts in the developing skeleton suggests that its role may be crucial for ossification and/or bone mass accrual. However, mice lacking *Esp* do not possess a skeletal or bone formation phenotype. Instead, they are defined by increased osteocalcin activity as a result of enhanced insulin signaling in the osteoblasts (Figure 1-6) (Ferron et al., 2010a; Lee et al., 2007).

By directly binding to, and dephosphorylating the insulin receptor in osteoblasts, ESP regulates the osteoblast expression of *Osteoprotogerin* to promote osteoclast maturation and the expression of osteoclast genes necessary for acidification of the resorption lacunae. Thus, through a triple inhibition pathway, ESP negatively regulates activity of osteocalcin and whole body glucose metabolism (Ferron et al., 2010a). As a result, $Esp^{-/-}$, as well as $Esp_{osb}^{-/-}$ (generated using *Col1a1*-Cre,) mice display marked hypoglycemia and hypoinsulinemia that is detected soon after birth and persists into adulthood. Glucose tolerance test and insulin tolerance tests indicate that $Esp^{-/-}$ mice have increased glucose tolerance as well as insulin sensitivity as a result of increased osteocalcin bioactivity (Lee et al., 2007).

ESP activity is regulated by at least two factors, the sympathetic tone and the osteoblast transcription factor ATF4. Its expression is dependent upon the β2-adrenergic receptor in osteoblasts, and accordingly, activated by the agonist isoproterenol (Yoshizawa et al., 2009). A decrease in *Esp* expression in osteoblasts, accompanied by the resulting increase osteocalcin levels, accounts for the increase in glucose tolerance observed in $Atf4^{-/-}$ mice (Hinoi et al., 2008).

However, ESP function is not evolutionarily conserved. Human ESP codes a shorter protein than mouse ESP, with which it shares 58% sequence identity. It lacks 12 exons and

several critical residues from its first phosphatase domain, including some within the PTP signature motif. This finding identifies ESP as the only class I classical protein tyrosine phosphatase that does not have a functional human homologue, and studies suggest that its function in murine osteoblasts does not extend to humans (Cousin et al., 2004b).

Instead, PTP1B replaces ESP function in human osteoblasts. PTP1B is expressed in the osteoblast and has been shown to bind to the human osteoblast insulin receptor *in vitro.* Interestingly, PTP1B is present at higher levels in human versus mouse osteoblasts, suggesting that its role in osteoblasts may be of more importance in human (Ferron et al., 2010a).

PTP1B

PTP1B (encoded by the gene *Ptpn1*) is a classical non-receptor protein tyrosine phosphatase that is a bona fide regulator of insulin receptor phosphorylation (Seely et al., 1996). Its C-terminus contains a predominantly hydrophobic domain that targets PTP1B to the cytoplasmic region of the endoplasmic reticulum so that it interacts with the insulin receptor upon its internalization.

To address whether this phosphatase is necessary to attenuate insulin action, mice lacking PTP1B were analyzed for their glucose response and insulin sensitivity. At 7-8 weeks of age, *Ptpn1*$^{-/-}$ mice display increased whole-body glucose tolerance and insulin sensitivity. These mice had decreased body weight and adiposity and were resistant to changes brought on by a high fat diet – they maintained body weight, adiposity, glucose, and insulin levels close to those of wild-type animals on a regular diet (Elchebly et al., 1999; Klaman et al., 2000).

Deletion of *Ptpn1* results in increased phosphorylation of the insulin receptor kinase domain specifically on tyrosines 1146, 1150, and 1551 in the muscle and liver, but not of white adipose tissue. These studies suggested PTP1B regulates whole body glucose homeostasis and

insulin sensitivity through regulation of insulin receptor phosphorylation in the insulin-responsive tissues of the muscle and liver (Elchebly et al., 1999; Klaman et al., 2000).

Thus, *Ptpn1*-specific deletions in the liver and muscle (using *Albumin*-Cre and *MCK*-Cre, respectively) were generated to confirm that PTP1B functions in these tissues to regulate glucose homeostasis. Both $Ptpn1_{liver}^{-/-}$ and $Ptpn1_{muscle}^{-/-}$ mice both display increased glucose tolerance (Zabolotny et al., 2008). $Ptpn1_{muscle}^{-/-}$ mice also display an increase in insulin sensitivity, as demonstrated by the insulin tolerance test and increased insulin receptor phosphorylation in these mice (Delibegovic et al., 2007a). Neither of these mice, however, displayed differences in adiposity or body weight change.

The same analysis was performed for $Ptpn1_{adipose}^{-/-}$ using *Adiponectin*-Cre and *aP2*-Cre. However, these mice, whether on a normal or high fat diet, did not exhibit any changes in body weight or adiposity (Bence et al., 2006b). The only significant difference in these mice was the size of their white adipocytes, which were significantly larger than normal, suggesting a possible role for PTP1B in lipogenesis. However, this change is not correlated with an increase in insulin signaling, suggesting that PTP1B regulates lipogenesis through an alternate pathway. This data demonstrated that PTP1B does not regulate whole body adiposity and weight through its expression in the white adipose (Owen et al., 2012).

The regulation of body weight and adiposity may instead rely on PTP1B actions in the brain, specifically in the POMC-neurons and neurons expression the leptin receptor. Deletion of PTP1B in the whole brain by *Nestin*-Cre recapitulated the body weight and adiposity observed in the total knockout, coupled with an increase in energy expenditure. In addition, these mice have increased leptin sensitivity, glucose tolerance, and insulin sensitivity. However, unlike that of the total knockout, $Ptpn1_{nes}^{-/-}$ mice have increased leptin levels (Bence et al., 2006b). This

function of PTP1B was dissected further by the generation of *Ptpn1* deletions in the POMC and leptin receptor-expression neurons of the brain. $Ptpn1_{POMC}^{-/-}$ mice have both decreased adiposity and body weight, while $Ptpn1_{LepR}^{-/-}$ mice display decreased body weight (they demonstrate decreased adiposity only under high fat diet conditions) (De Jonghe et al., 2011; Tsou and Bence, 2012; Tsou et al., 2012). These results suggest that PTP1B functions in this region of the brain to regulate leptin sensitivity and energy expenditure, thus affecting body weight and adiposity.

TC-PTP

Sharing 74% catalytic domain (72% identity, 86% similarity) homology with PTP1B, T-cell protein tyrosine phosphatase (TC-PTP, encoded by the gene *Ptpn2*) is a non-receptor classical PTP that exists in two isoforms. The longer, 48-kDa form (TC48) is restricted to the ER while the 45-kDa (TC45) form, which lacks the hydrophobic C-terminus, is localized to the nucleus. Though TC-PTP exists mainly as TC45, it has been demonstrated that its localization is not limited to the nucleus, and that TC45 can shuttle between the nucleus and cytoplasm. In response to insulin stimulation, TC45 exits the nucleus where it accumulates in the cytoplasm to interact with the internalized insulin receptor to attenuate its phosphorylation (Galic et al., 2003). Single nucleotide polymorphisms (SNPs) in human *PTPN2* have been associated with susceptibility for the development of type 1 diabetes, Crohn's disease, rheumatoid arthritis, and juvenile idiopathic arthritis (Espino-Paisan et al., 2011; 2007; Smyth et al., 2008; Thompson et al., 2010).

Due to its function in immune homeostasis, mice that lack TC-PTP develop systemic inflammatory disease and do not survive past 5 weeks of age (You-Ten et al., 1997). During this time, they display growth retardation, splenomegaly, lymphadenopathy and an inability to respond to mitogens and T-cell dependent B-cell responses. These mice have increased

mononuclear infiltrates and cytokine production in the liver and salivary gland, which leads to tissue damage and their eventual death (Heinonen et al., 2004). TC-PTP function has been somewhat elucidated in T-cells, where TC-PTP directly binds to and dephosphorylates Src family kinases to regulate T cell activation and proliferation (Wiede et al., 2011). Mice lacking *Ptpn2* specifically in T-cells develop whole-body inflammation and autoimmunity (Heinonen et al., 2004).

Due to their growth defects, *Ptpn2*$^{-/-}$ mice have smaller skeletons with decreased vertebrate and femoral length. They also display higher levels of bone resorption, coinciding with significantly larger osteoclasts. *Ptpn2*$^{-/-}$ bone-marrow osteoclast precursors grown *ex vivo* in the presence of M-CSF and RANKL also develop into larger osteoclasts, suggesting that TC-PTP regulates osteoclast fusion and/or maturation cell-autonomously. The *Ptpn2* knockout skeletal phenotype is somewhat strain-dependent, as mice on a BALB/c background had smaller skeletons with increased trabecular bone volume. On a C57BL/6 background, the skeletons displayed no difference in trabecular bone volume and lifespan was increased (Doody et al., 2012).

The early lethality of *Ptpn2*$^{-/-}$ prevents analysis of its role in whole body metabolism. Yet *Ptpn2*$^{+/-}$ mice survive and display no obvious abnormalities. Their body weights are similar to those of their wild-type littermates, suggesting that one allele of *Ptpn2* is sufficient to normalize cytokine production and the inflammatory response. Though they display normal glucose tolerance and insulin sensitivity, mice lacking one allele of *Ptpn2* have decreased liver glucose output. Lack of one allele of *Ptpn2* also imparts protection from high fat diet induced hyperglycemia. In the hepatocytes isolated from *Ptpn2*$^{+/-}$ mice, both Y1162/Y1163 insulin receptor and IL-6 induced STAT3 phosphorylation were increased, coinciding with decreased

expression of the gluconeogenic genes *G6pc* and *Pck1*. This suggests that TC-PTP regulates insulin signaling in the liver *in vivo* (Fukushima et al., 2010).

To assess the role of TC-PTP in the muscle, $Ptpn2_{muscle}^{-/-}$ mice were generated using a floxed allele of *Ptpn2* crossed to *MCK*-Cre. These mice displayed no difference in insulin signaling in the muscle, and thus conferred no alternations in glucose homeostasis. This held true even when $Ptpn2_{muscle}^{-/-}$ mice were challenged on a high fat diet, suggesting that TC-PTP does not impart its regulation of the insulin receptor in muscle cells (Loh et al., 2012).

It has been demonstrated that levels of TC-PTP were increased in the hypothalamus upon leptin treatment or diet-induced hyperleptinemia, suggesting that TC-PTP regulates leptin signaling or is regulated by leptin signaling. To elucidate the function of TC-PTP in the brain, $Ptpn2_{Neuron}^{-/-}$ mice were generated using *Nestin*-Cre. The administration of leptin resulted in increased STAT3 Y705 in the hypothalamus, coinciding with increased expression of STAT3 gene targets *Pomc* and *Agrp*. Using overexpression and knockdown analysis of TC-PTP in Chinese hamster ovary (CHO) cells, Koh et al. demonstrated that TC45 regulates leptin-induced STAT3 Y705 phosphorylation. Expression of the substrate-trapping mutant (TC45-D182A) results in nuclear accumulation of Y705-phosphorylated STAT3, supporting the notion that TC45 acts on STAT3 in the nucleus (Loh et al., 2011a).

$Ptpn2_{Neuron}^{-/-}$ mice were approximately 20% smaller, coinciding with decreased growth hormone and IGF-1 levels, decreased food intake, and increased energy expenditure. They displayed increased glucose tolerance and insulin sensitivity with reduced fasted glucose and insulin levels. Lack of TC-PTP in neuronal cells confers a resistance to diet-induced obesity (Loh et al., 2011a).

Figures

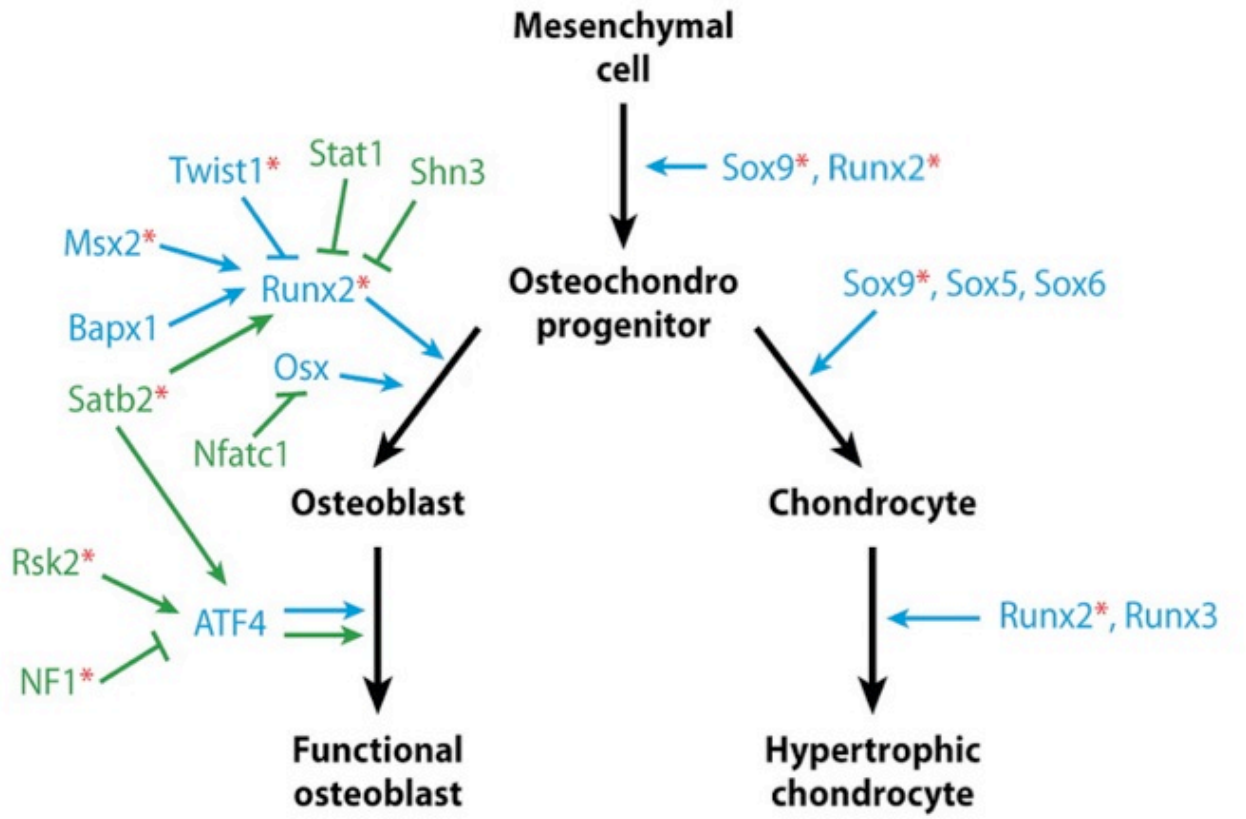

Figure 1-1. A schematic representation of the transcriptional control of the chondrocyte and osteoblast lineages Regulation at the transcriptional level is displayed in blue, while regulation at the post-transcriptional level is shown in green. Red asterisks indicate genes that have been shown to be related to skeletal disease in humans. Figure adapted from (Karsenty, 2008)

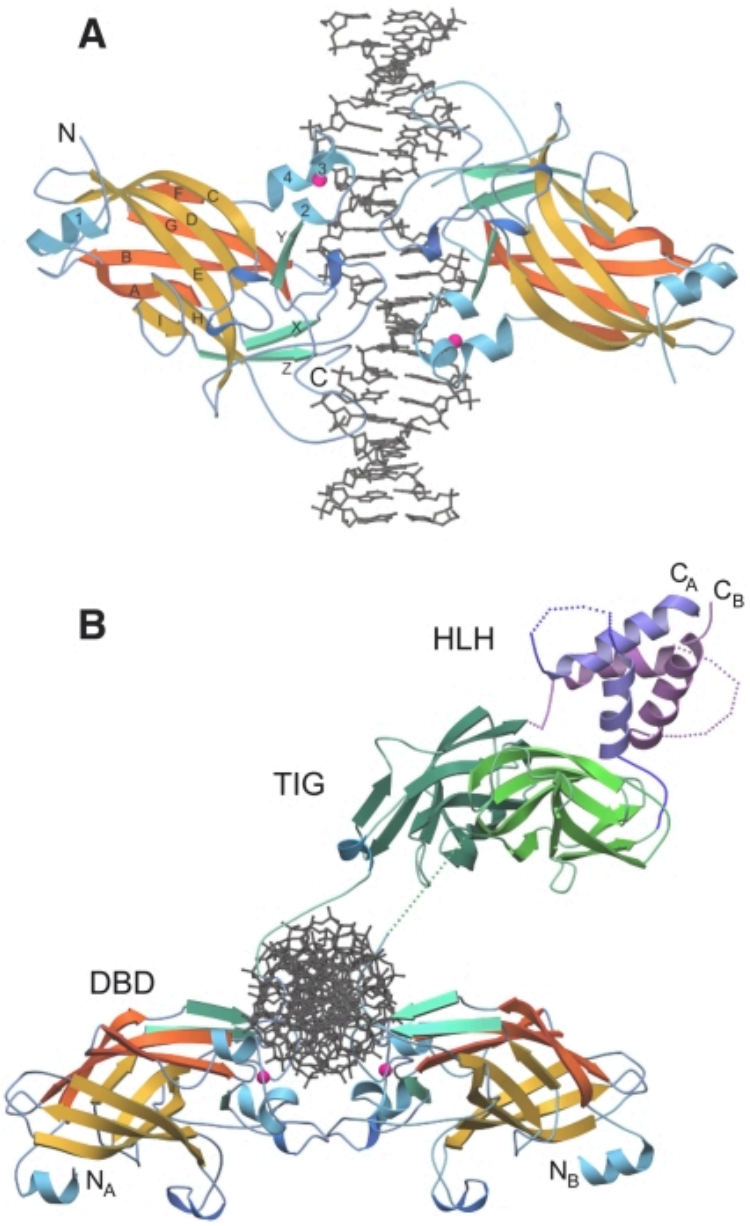

Figure 1-2. Crystal structure of Ebf1 dimer bound to DNA from two different views. A. β strands of the DNA binding domains (DBDs) bound to DNA. **B.** Aerial view of the dimers, including the immunoglobin (TIG), and helix-loop-helix domain (HLH). (Treiber et al., 2010a)

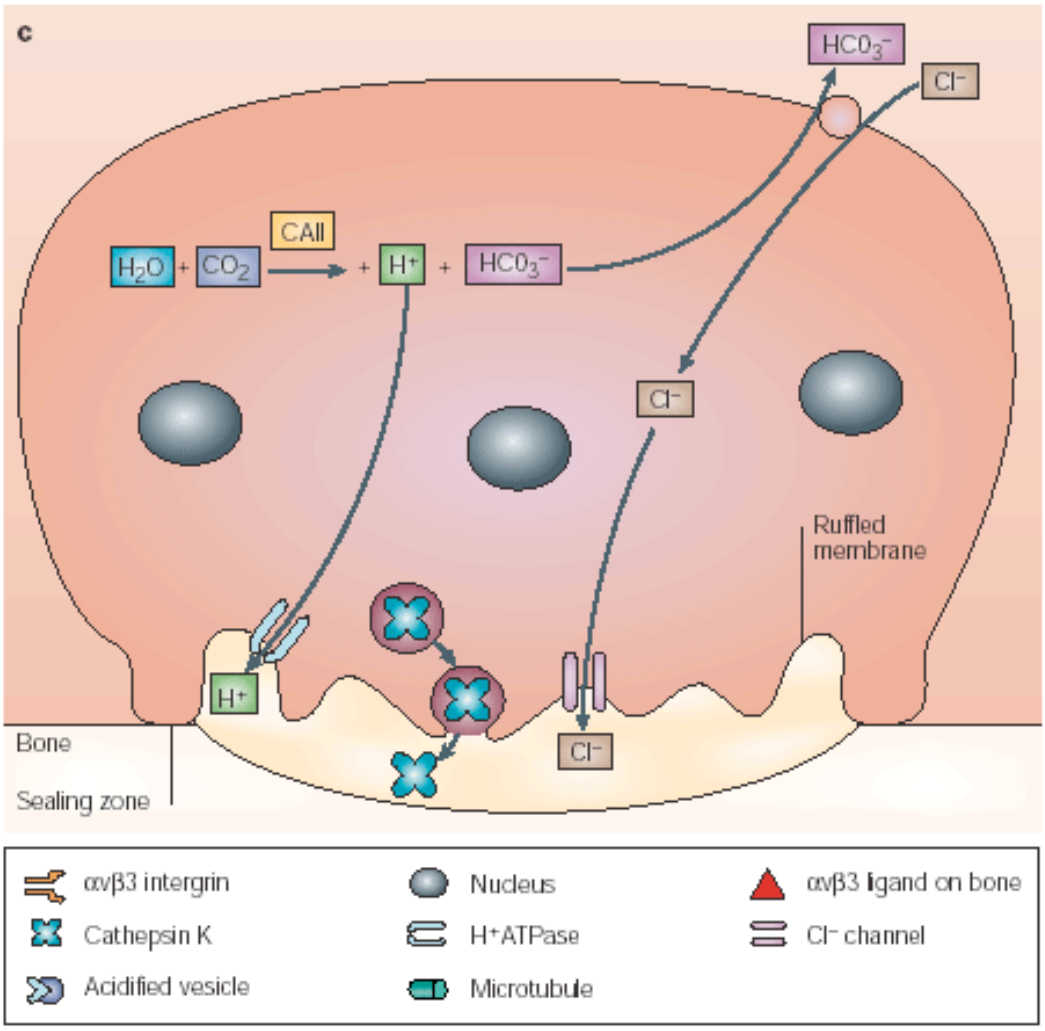

Figure 1-3. The multinucleated osteoclast forms an acidic lacuna that facilitates the resorption of bone matrix. Figure from (Teitelbaum and Ross, 2003)

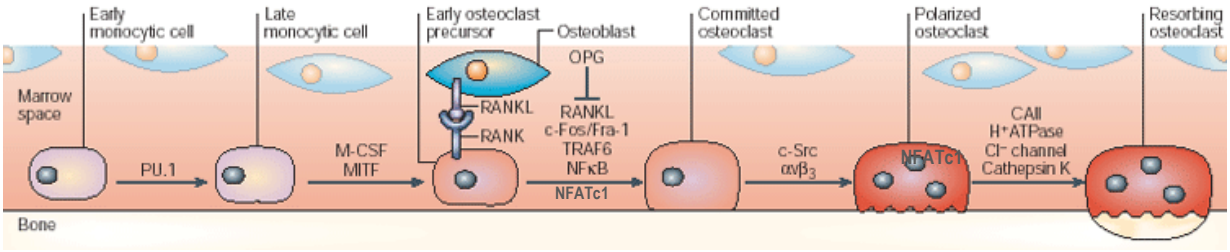

Figure 1-4. Molecular mechanisms regulating osteoclast differentiation. Figure adapted from (Teitelbaum and Ross, 2003)

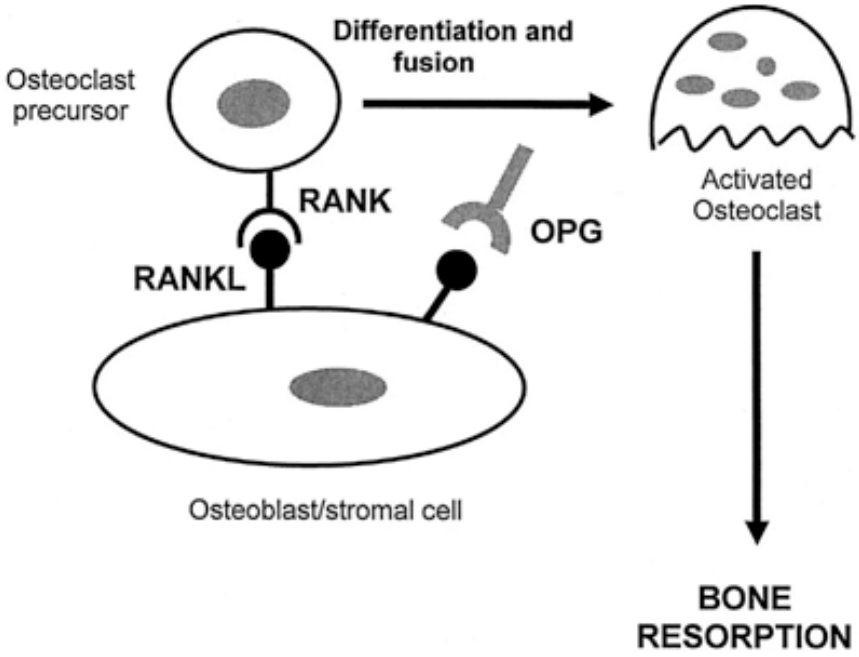

Figure 1-5. The RANKL/OPG/RANK system. Osteoblasts secrete OPG, is a soluble RANK decoy receptor preventing RANKL-stimulation of osteoclast maturation. Figure from (Yasuda et al., 1998b)

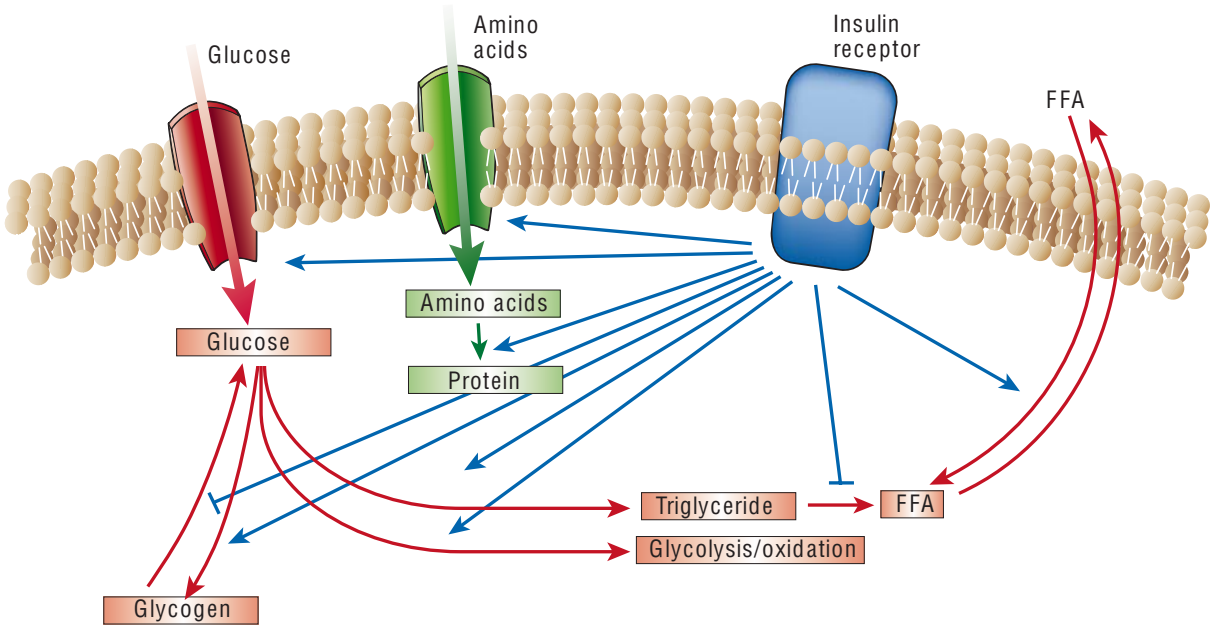

Figure 1-6. Insulin signaling in the cell activates uptake of glucose, amino acids, and free fatty acids. Figure from (Saltiel and Kahn, 2001).

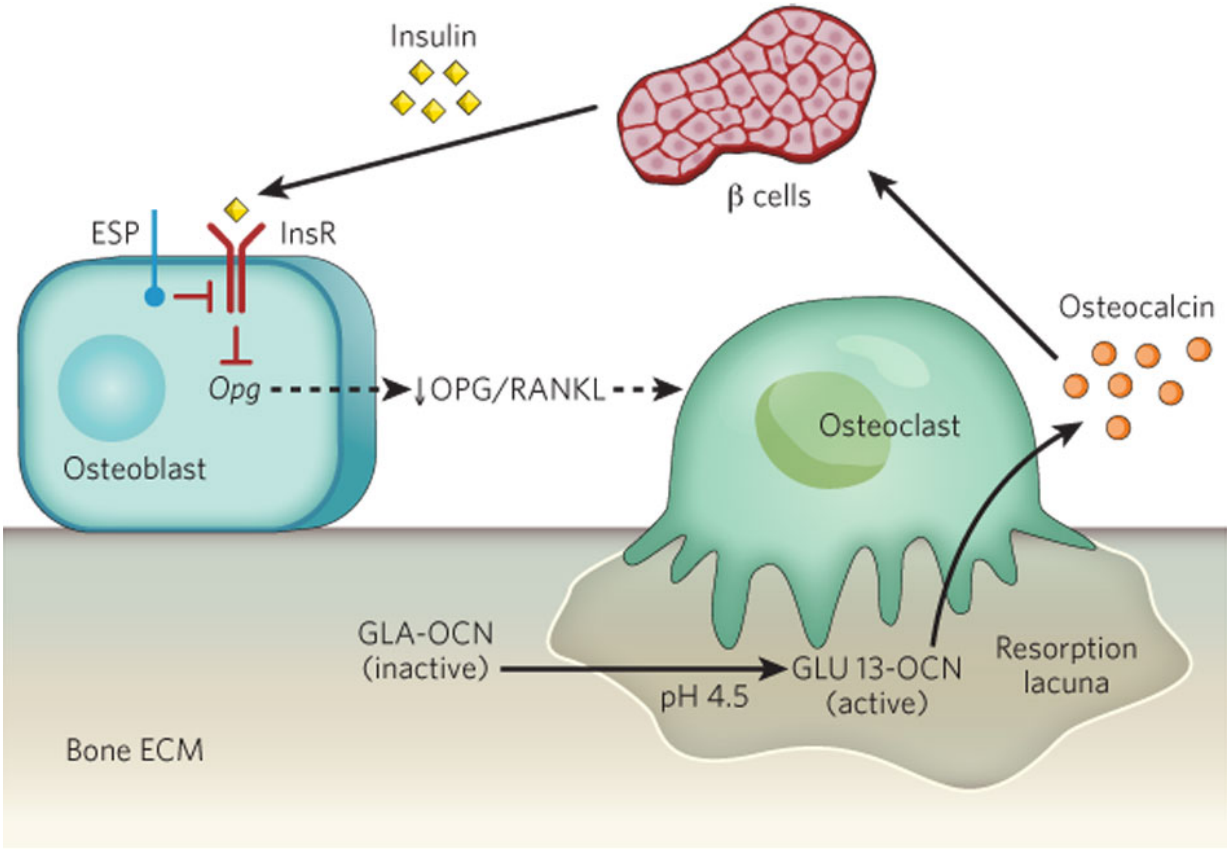

Figure 1-7. Bone activation of osteocalcin activity. Insulin signals to the osteoblast, repressing *Opg* expression and the activation of osteoclast maturation. Mature osteoclasts form an acidic resorption lacuna that facilitates the release and decarboxylation of osteocalcin, which when released into circulation, promotes insulin secretion in the pancreas. Figure from (Ferron et al., 2010a)

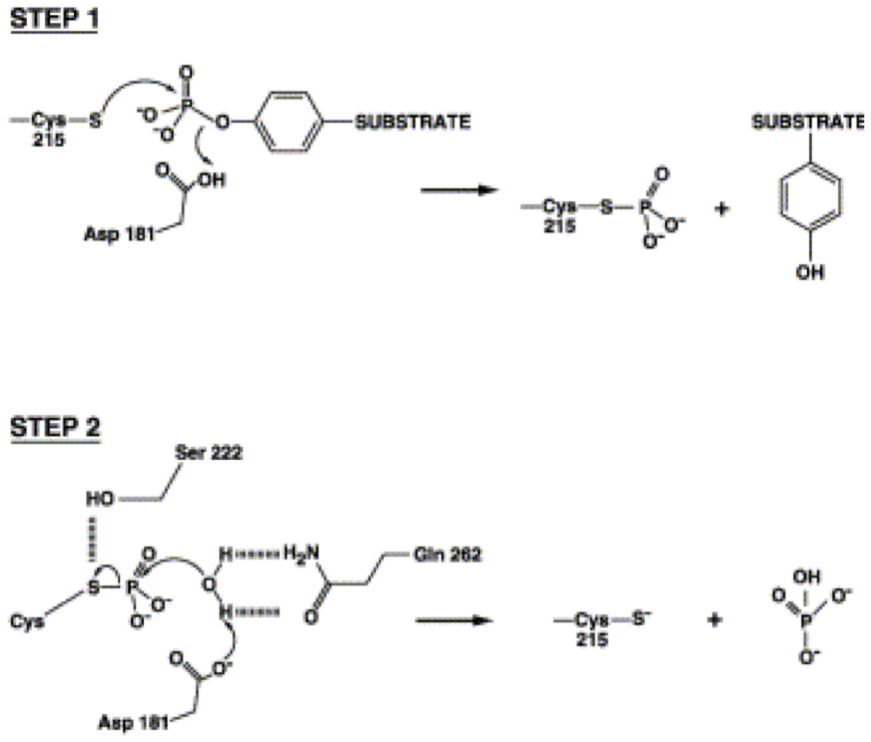

Figure 1-8. The protein tyrosine phosphatase catalytic mechanism. The first step of catalysis involves nucleophilic attack of the phosphorylated substrate and transfer of the phosphotyrosyl group to the PTP. The substrate is thus released. In the second step, the phosphotyrosyl group is protonated and released by a basic aspartic acid group. Figure from (Tonks, 2003)

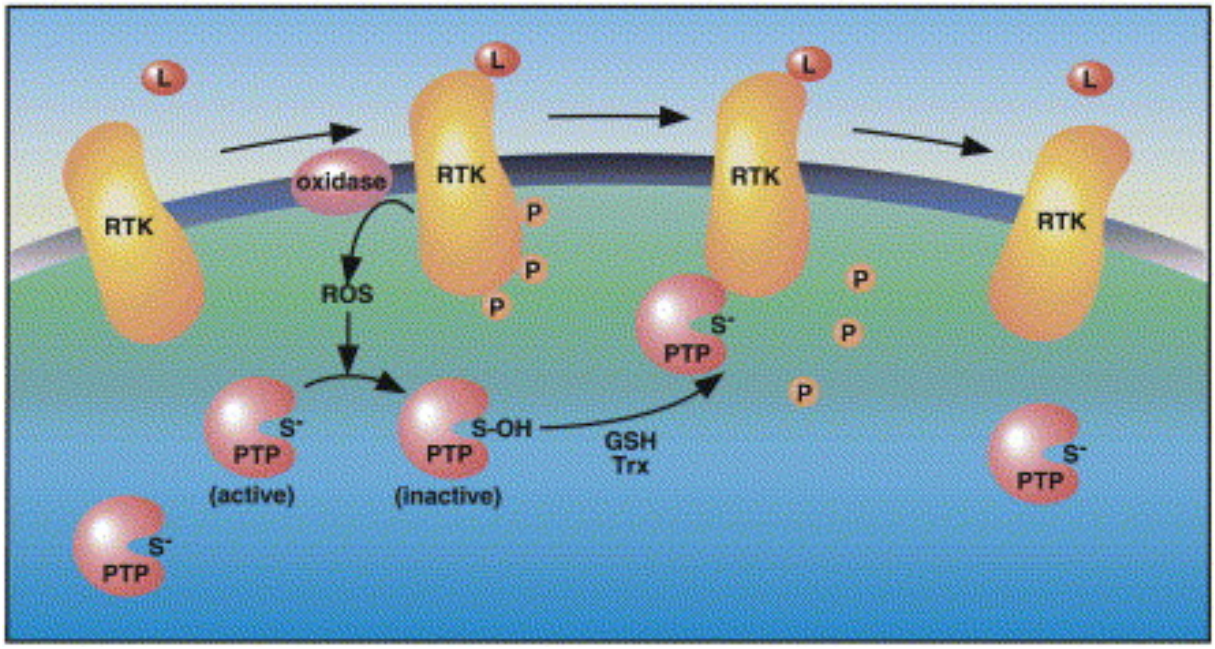

Figure 1-9. A model of protein tyrosine phosphatase activity. Ligand binding activates an NAD-dependent oxidase, which reversibly oxidizes the catalytic cysteine of an active PTP. Inactivation of the PTP thus allows phosphorylation of the RTK substrate and signalling to proceed. Figure from (Tonks, 2003)

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Chapter Two: The transcription factor early B-cell factor 1 regulates bone formation in an osteoblast-nonautonomous manner

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Preface

The completion of this work is owed to the contribution of many people, including those represented on the authorship list, and the help of others listed below. Ildiko Gyory generated the *Ebf1*$_{fl/fl}$ mice. *Runx2*-Cre mice were generated and provided by Dr. Jan P. Tuckermann. *Ebf1*$_{fl/fl}$*;Runx2-Cre* mice were generated and maintained in a joint effort by Munevver Parla Makinistoglu and myself. Sören Boller performed ChIP analysis on 10TT1/2 cells. Dr. Rudolf Grosschedl and Dr. Gerard Karsenty conceived the study. We thank Dr. Sebastian Pott for bioinformatics analysis and comparison of the Ebf1 peaks with DNAse I hypersensitivity, and Dr. Patricia Ducy for critical reading of the manuscript. Under the mentorship of Dr. Gerard Karsenty, I performed the rest of the work. This work was supported by the National Institutes of Health training grant Hormones: Biochemistry and Molecular Biology – T32DK07328 (T.Z.) and grant from the National Institutes of Health (G.K.)

Summary

Early B-cell factor 1 (Ebf1) is a transcription factor whose inactivation in all cells results in high bone mass because of an increase in bone formation. This observation suggests Ebf1 may be an inhibitor of osteoblast differentiation. To test this contention, we analyzed *Ebf1* pattern of expression and function in osteoblasts in *ex vivo* and *in vivo* through osteoblast-specific inactivation in in the mouse. We show here that *in vivo* deletion of *Ebf1* in osteoblast progenitors does not affect osteoblast differentiation or bone formation accrual post-natally. These observations indicate that the phenotype described in *Ebf1*$^{-/-}$ mice is not osteoblast-autonomous

Introduction

Our understanding of the transcriptional control of the differentiation processes that generate, during embryonic development, the various cell types of mesenchymal origin, i.e. the osteoblast, chondrocyte, myoblast, and adipocyte, has made considerable progress during the past two decades. Although these cell types derive from a common progenitor, the mesenchymal stem cell (MSC), their differentiation along various lineages depends on distinct sets of transcription factors. In particular, the early steps of differentiation of MSCs to osteoblasts require the function of Runx2, while later steps of differentiation involve at least two other transcription factors, Osterix and Atf4 (Karsenty et al., 2009).

Besides the cardinal transcription factors mentioned above, many others have been shown to affect osteoblast differentiation either by modulating *Runx2* activity or by functioning independently of *Runx2*. Twist and Schnurri-2 are two examples of the former (Bialek et al., 2004; Jones et al., 2007), while members of the AP-1 family and Creb act independently of *Runx2* to affect osteoblast differentiation and proliferation (Bozec et al., 2010; Kajimura et al., 2011). Another factor for which mouse genetics has given clear indications that it contributes to osteoblast phenotype is Ebf1.

Early B-cell factor 1 (Ebf1) is a member of a small family of transcription factors that contains an atyptical zinc finger DNA binding domain and a non-basic helix-loop-helix (HLH) dimerization domain (Hagman et al., 1993). Originally cloned as a putative B-cell transcription factor, Ebf1 was confirmed by loss-of-function experiments in the mouse to be a pioneer factor essential for the commitment and maintenance of B-cell fate (Gyory et al., 2012; Hagman et al., 1993). The cell differentiation ability of Ebf1 has been expanded to neurons of the embryonic striatum during mouse development (Garel et al., 1999). More recently, two indirect but

convergent lines of evidence raised the prospect that Ebf1 may prevent osteoblast differentiation and instead favor allocation of MSCs toward the adipocyte lineage. First, in the pre-adipogenic 3T3L1 cell line Ebf1 promotes *PPARγ* expression, while reducing *Ebf1* expression inhibits the adipocyte differentiation potential of this cell line (Akerblad et al., 2002; Jimenez et al., 2007). Second, and more importantly for our purpose, analysis of mice lacking *Ebf1* in all cells revealed a marked increase in the number of osteoblasts and in bone formation parameters (Fretz et al., 2010; Hesslein et al., 2009). This experiment established that Ebf1 as a negative regulator of osteoblast differentiation. As importantly, that this high bone mass phenotype was observed in mice lacking *Ebf1,* but not other members of this small family of transcription factors, indicated that there was no overt redundancy between Ebf1 and other members of the Ebf family when it comes to the regulation of osteoblast differentiation.

In view of the strong phenotype of *Ebf1*$^{-/-}$ mice and of Ebf1 differentiation ability in other cell lineages, it is legitimate to suspect that *Ebf1* inhibits osteoblast differentiation in a cell-autonomous manner. To determine if this is indeed the case we relied on Ebf1 knockdown in cell culture and the analysis of mutant mice lacking *Ebf1* only in cells of the osteoblast lineage. To our surprise, *Ebf1* deletion in cells of the osteoblast lineage throughout development and after birth has no overt deleterious consequences on the differentiation of osteoblasts, bone formation parameters, or overall bone mass accrual. Taken at face value, these observations identify Ebf1 as one of the few transcription factors inhibiting osteoblast differentiation in a non cell-autonomous manner.

Materials and Methods

Mice Generation. *Ebf1*$^{fl/+}$mice were generated as previously described (Treiber et al., 2010b). To generate osteoblast-specific *Ebf1* deficient mice, *Ebf*$^{fl/fl}$ mice were crossed with *Runx2- Ebf1* $^{fl/+}$;*Cre* mice, which were then crossed to *Ebf*$^{fl/fl}$ mice to generate *Ebf1*$_{osb}^{-/-}$ mice. Genotypes of mice were determined by PCR. Primer sequences are available upon request. All mice were 1-month old males maintained on the C57BL/6 genetic background.

In situ hybridization. Embryos were fixed in 4% paraformaldehyde and embedded in paraffin. Radioactive in situ hybridization was performed on 6μm sections using ^{35}S-labeled riboprobes. Hybridizations were performed overnight at 55°C, and washes were performed at 63°C. Autoradiography and Hoechst 33528 staining were performed as described (Ducy and Karsenty, 1995). Probe sequences available upon request.

Molecular Studies. RNA isolation and quantitative PCR were performed following standard protocols.

Statistical Analysis. Results are given as means ± standard error of means unless otherwise indicated. Statistical analyses were performed using the Student's t-test

Cell Culture. Primary osteoblasts were cultured and differentiated as previously described (Ducy et al., 2000). Cells were transfected with siRNA pools (on-TARGETplus SMARTpool, Dharmacon) according to manufacturer instructions. *Ebf1*$^{+/+}$ or *Ebf1*$^{-/-}$ osteoblasts were generated by infecting *Ebf1*$^{fl/fl}$ osteoblasts with either green fluorescent protein (GFP)- or Cre-expressing adenovirus (University of Iowa)

Chromatin Immunoprecipitation (ChIP). Target cells were harvested and resuspended in medium at a concentration of 1 to 2x10^6 cells/ml. Crosslinking mix (11%% formaldehyde, 100mM NaCl, 0.5mM EGTA, 1mM EDTA, 50mM Hepes pH 8.0) was added to a final concentration of 1% formaldehyde. Crosslinking reaction was stopped after 10min at room temperature by the addition of glycine to a final concentration of 125mM. Cells were spun down immediately and washed three times with ice cold PBS. They were resupended in lysis buffer (1.25% SDS, 12.5 mM EDTA, 62.5mM Tris-HCl pH 8.0, protease inhibitor mix) with a concentration of 20 to 40x10^6 cells/ml. With a Bioruptor® Standard the chromatin was sheared into pieces of 300 to 500 bp. This chromatin was stored at -80°C and subsequently used for ChIP experiments.

For each ChIP 100µl chromatin were diluted 1/10 in dilution buffer (50mM Tris-HCl pH 8.0, 5mM EDTA, 200mM NaCl, 0.5% NP-40, protease inhibitor mix). 4µg of an anti-murine Ebf1 antibody (noncommercial polyclonal rabbit anti Ebf1 antibody, protein G purified) or normal rabbit IgG (Millipore 12-370) was added. Samples were rotated for 16-20 hours at 4 °C and washed Protein-A Sepharose beads were added for another two hours. Subsequently beads were washed five times with wash buffer (0.1% SDS, 1% NP-40, 2mM EDTA, 500mM NaCl, 20mM Tris-HCl pH 8.0) and four times with TE buffer (10mM Tris-HCl pH8, 1mM EDTA pH8.0). Immunoprecipitated chromatin was eluted two times with 50µl elution buffer (2%SDS, 10mM Tris-HCl pH8, 1mM EDTA pH8.0) for 10min at 65°C shaking. Elutions were pooled and decrosslinked for at least six hours at 65°C. DNA was finally purified with QIAquick® PCR Purification Kit (Qiagen Cat.No.28106). The purified DNA was used in quantitative PCR to analyze binding of Ebf1 to DNA.

Histology. Static and dynamic histomorphetric analyses were performed on vertebral column specimens collected from 1-month old mice using undecalcified sections according to standard protocols, and using the Osteomeasure analysis system (Osteometrics).

Results

Ebf1 is expressed at low levels in osteoblasts during embryonic development

At the onset of this study to guide our investigation, we sought to determine which members of the *Ebf* family were the most highly expressed in primary osteoblasts. qPCR analysis using exonic primers normalized to genomic DNA revealed that in osteoblasts, *Ebf1* was clearly more abundantly expressed than the other three members of the *Ebf* family. In cells that that were fully differentiated, this difference in level of expression was at least one order of magnitude (Fig. 2-1A). Given this result and the important fact that deletion of *Ebf1* in all cells in and by itself suffices to affect bone mass accrual, we thus focused the remainder of our analysis on the function *Ebf1* may have in osteoblasts.

We first studied *Ebf1* pattern of expression in developing skeleton by *in situ* hybridization. As previously shown, the marker of bone formation *Runx2*, is robustly expressed in osteoprogenitors of the developing ribs already at E12.5 (Ducy et al., 1997), In contrast, in an adjacent section, the expression of *Ebf1* in osteoprogenitors, although detectable, was significantly weaker (Fig. 2-1B). Similarly, at E14.5 and E16.5, expression of *Runx2* in cells of the osteoblast lineage was quite high, but expression of *Ebf1* remained barely above the limit of detection (Fig. 2-1B). Finally, we compared the expression of *Ebf1* in adult tissue, and also observed rather low expression in bone and cartilage (Fig. 2-1C). In summary, these results

indicate that *Ebf1* expression does not appear as early as *Runx2* and is less pronounced than the one *Runx2* during skeletogenesis.

Ebf1 affects osteoblast gene expression ex vivo.

Next, to determine whether or not Ebf1 may be a cell-autonomous molecular suppressor of osteoblast differentiation, we analyzed the effects of siRNA-mediated down-regulation of *Ebf1* mouse osteoblasts that were transfected with a siRNA to transiently suppress *Ebf1* expression but not affect *Ebf2, 3,* or *4* expression (Fig. 2-2A).

For that purpose, primary osteoblasts from calvaria of newborn mice were transfected with *Ebf1* siRNA, resulting in a 76% decrease in *Ebf1* expression. We also confirmed that the siRNA we used was specifically targeting *Ebf1*, and did not result in altered expression of *Ebf2, 3,* or *4* (Fig. 2-2A). *Ebf1* knockdown in primary osteoblasts increased expression of *Osterix, Col1a1*, *Osteocalcin*, and *Alkaline phosphatase* (*Alpl*). These results were certainly consistent with the notion that Ebf1 acts as a cell-autonomous inhibitor of osteoblast differentiation. Of note, within the conditions of this experiment, we did not observe any change in the expression of adipocyte-specific transcription factors such as *PPARγ, C/EBPα, C/EBPβ* (Fig. 2-2A).

In view of these results we asked whether Ebf1 directly binds to some of the genes whose expression was perturbed by its knockdown in the above-mentioned experiment. We employed an Ebf1 chromatin immunoprecipitation (ChIP) seq analysis to identify potential Ebf1-binding sites, and further validated these sites by quantitative ChIP. This analysis indicated that Ebf1 binds at multiple sites in the *Alpl* and *Osterix* regulatory regions (Fig. 2-2B). It is interesting to note that the Ebf1-bound site on the *Alpl* gene coincides rather well with the presence of DNase hypersentitive sites in mesenchymal cells but not in B cells (Fig. 2-2C).

Ebf1-independent osteoblast differentiation in vivo

In view of this encouraging set of *ex vivo* observations, we next studied the function of Ebf1 specifically in osteoblasts *in vivo* and crossed $Ebf1^{fl/fl}$ mice with mice expressing *Cre* recombinase under the control of the *Runx2* regulatory elements (Rauch et al., 2010). We chose *Runx2-Cre* mice to address this question, as *Runx2* is the earliest and also the most specific molecular marker of the osteoblast lineage identified to date (Ducy et al., 1997). Hence, this *Cre* driver would allow us to study *Ebf1* functions in cells of the osteoblast lineage at each stage of differentiation. Prior to analyzing these mice, we verified that we had achieved efficient deletion of *Ebf1* in osteoblasts. *Ebf1* was decreased by 80% in whole bone, and by more than 95% in osteoblasts derived from bone marrow. Although *Ebf1* expression was also reduced in cartilage, its expression was not affected in the other tissues tested (Supplemental Fig. 2-1).

$Ebf1_{osb}^{-/-}$ mice were born at the expected Mendelian ratio, had normal life expectancy, and appeared overall normal, indicating that its expression in *Runx2*-expressing cells is dispensable for normal embryonic development. Body weight, epididymal fat pad weight, body and femoral lengths were similar between $Ebf1_{osb}^{-/-}$ mice and control littermates ($Ebf1^{fl/+}$, $Ebf1^{fl/fl}$, and *Runx2-Cre* mice) (Fig. 2-3A). To our surprise, bone histomorphometric analysis performed in vertebrae of 1-month old $Ebf1_{osb}^{-/-}$ and control mice did not reveal any change in osteoblast number, bone formation rate, and bone mass (Fig. 2-3B). Serum osteocalcin levels were similarly unaffected in $Ebf1_{osb}^{-/-}$ mice (Fig. 2-3C). Gene expression analysis performed in bone tissue did not record any of the changes in gene expression that had been observed in cell culture experiments (Fig. 2-3D). This set of observations indicates that *in vivo*, Ebf1 does not regulate

osteoblast differentiation through its expression in cells of the osteoblast lineage even though *ex vivo Ebf1*$^{-/-}$ osteoblasts form less mineralization nodules and produce slightly less alkaline phosphatase (Fig. 2-4A and 2-4B).

Discussion

Previous observations stemming from cell culture experiments and from the analysis of mice lacking *Ebf1* in all cells had indicated that Ebf1 is a negative regulator of osteoblast differentiation and bone formation (Akerblad et al., 2002; Hesslein et al., 2009; Jimenez et al., 2007). These data immediately raised the question of whether Ebf1 acts in a cell-autonomous manner to fulfill this function. This is an even more important question given the fact that among all members of this small family of transcription factors, *Ebf1* is the most highly expressed in cells of the osteoblast lineage by far.

Transient *Ebf1* loss of function experiments performed in primary osteoblasts fully supported the notion that Ebf1 negatively regulates osteoblast-specific gene expression. Since expression of important genes for the osteoblast phenotype as *Osterix, Col1a1, Osteocalcin*, and *Alpl* were dramatically increased in cells lacking *Ebf1*. These results were fully consistent with the high bone mass observed in *Ebf1*$^{-/-}$ and suggested that Ebf1 acts in a cell autonomous manner to regulate osteoblast differentiation.

Hence, it came as a surprise that cell-specific *Ebf1* deletion in cells of the osteoblast lineage in the mouse does not have any overt consequences on osteoblast differentiation *in vivo*. Results of this analysis in *Ebf1*$_{osb}^{-/-}$ mouse model are surprising for several reasons.

The first reason is that the *in vivo* results have no clear relationship to what was previously observed in cell culture by others and by us, after a decrease of *Ebf1* expression in

differentiated osteoblasts (Akerblad et al., 2002; Jimenez et al., 2007). As such, these results illustrate how cautious one should be when making inference about the differentiation ability of a given transcription factor or of any other regulatory gene based on cell culture assays alone. This result was even less expected given the fact that Ebf1 binding sites are present in such an important regulator of osteoblast differentiation as *Osterix*. Last, but not least, these results were also unanticipated because they are not consistent with what has been observed in mice globally lacking *Ebf1*, which display high bone mass with a concomitant decrease in adiposity (Hesslein et al., 2009).

Although the formal possibility remains that *Ebf1* function is masked by the remaining expression of *Ebfs 2, 3,* and *4*. We note that mice lacking *Ebf1* in all cells present cell differentiation defects even though other *Ebfs* are normally expressed. Thus we believe this is an unlikely possibility (Lin and Grosschedl, 1995). At the present time the most likely interpretation of the unanticipated results presented here is that the phenotype observed previously in *Ebf1*$^{-/-}$ mice may be due to a cell-nonautonomous defect. Hence, *Ebf1* may belong to a novel class of transcriptional regulators of osteoblast differentiation that acts in a non-cell autonomous manner.

Acknowledgements

We thank Dr. Sebastian Pott for bioinformatic analysis and comparison of the Ebf1 peaks with DNaseI hypersensitivity, and Dr. Patricia Ducy for critical reading of the manuscript. This work was supported by the National Institutes of Health training grant Hormones: Biochemistry and Molecular Biology – T32DK07328 (T.Z.) and grant from the National Institutes of Health (G.K.).

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Figures

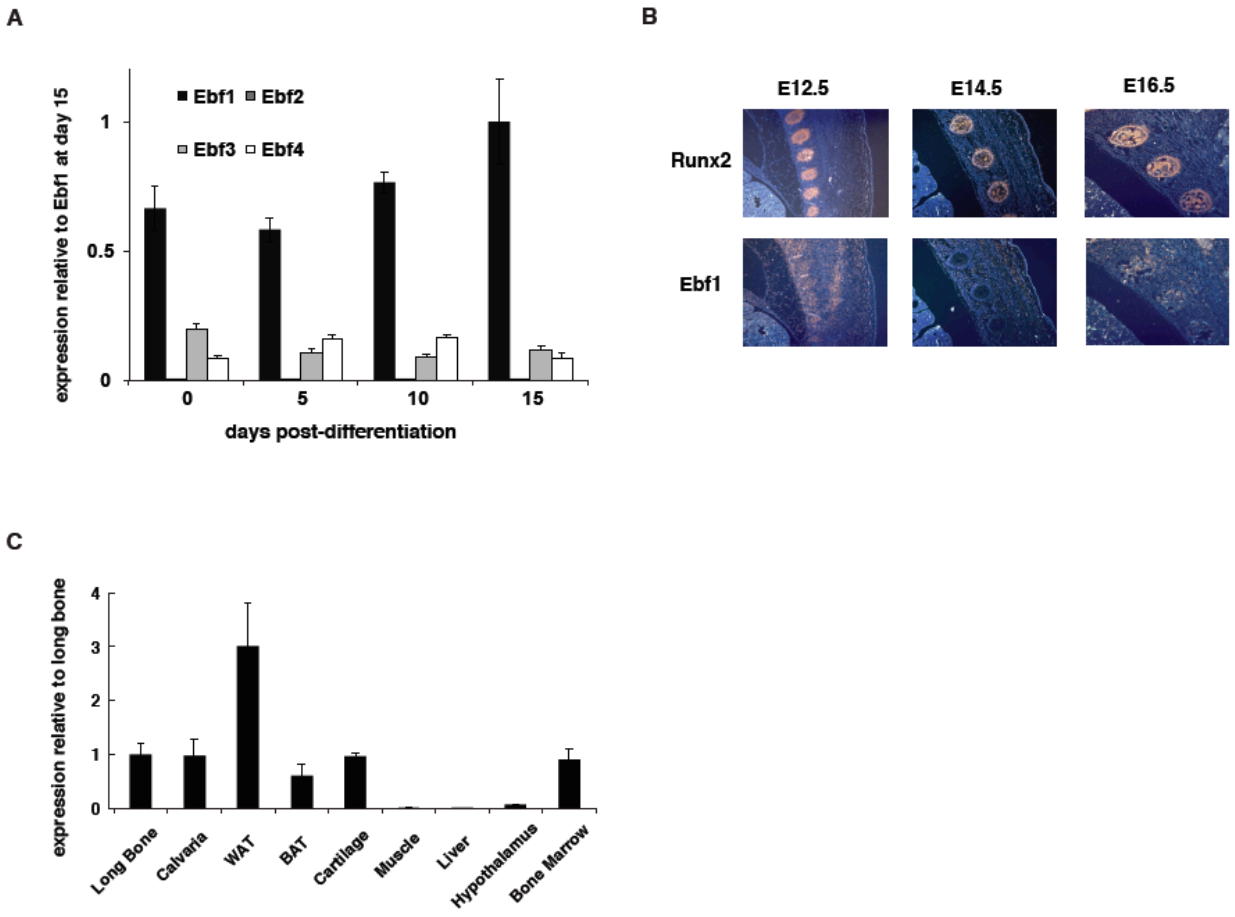


Figure 2-1. Ebf1 expression analysis. (**A**) Normalized expression of *Ebf1, 2, 3,* and *4* by qPCR in primary osteoblasts differentiated in culture. (**B**) Expression pattern analysis of *Ebf1* by radioactive *in situ* hybridization of adjacent sections featuring osteoblast (*Runx2*) regions in mouse embryos. (**C**) qPCR analysis of Ebf1 expression in adult mouse tissue. Reference gene is GAPDH. White adipose tissue, WAT. Brown adipose tissue, BAT.

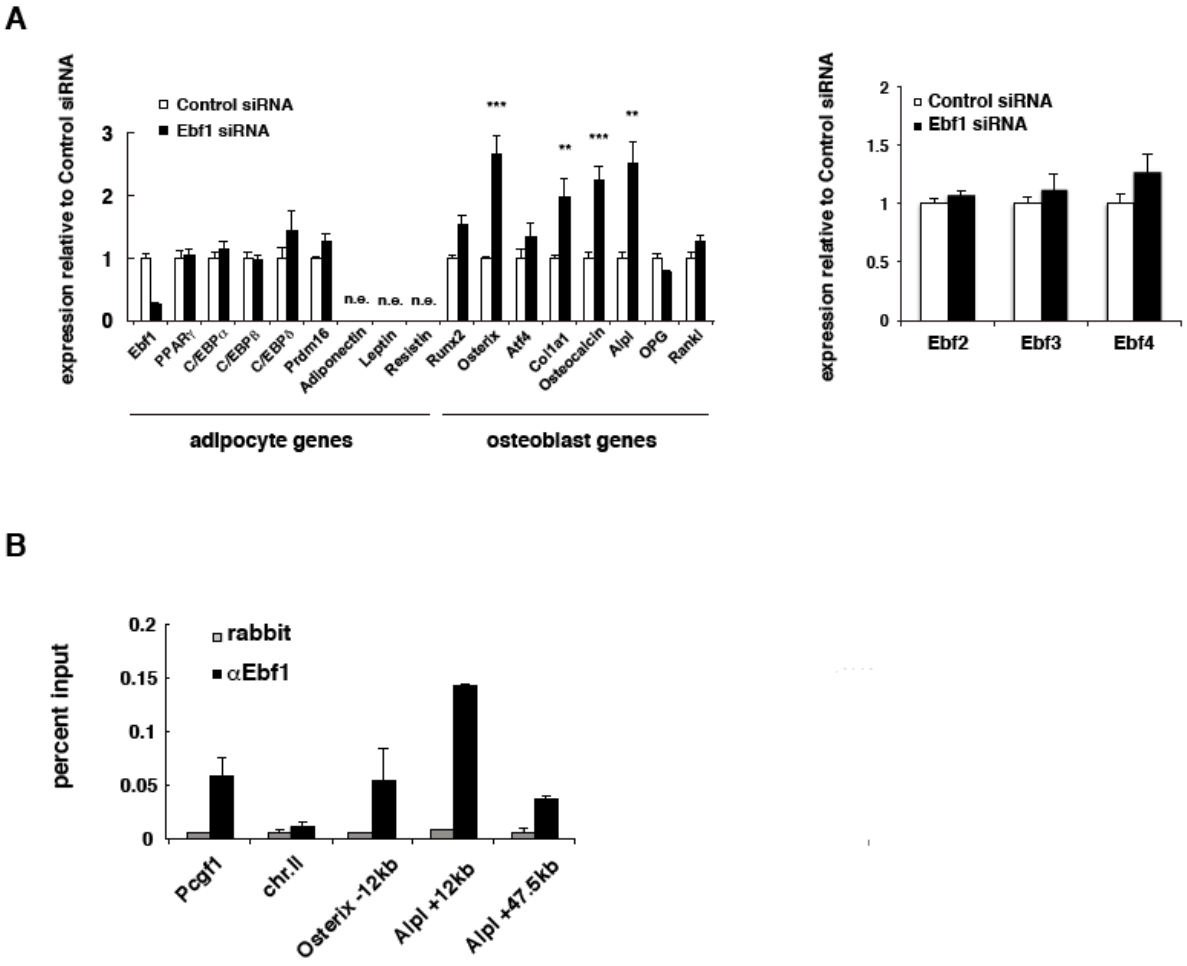


Figure 2-2. Ebf1 analysis in 10T1/2 cells (**A**) Ebf1 siRNA knockdown in primary osteoblasts. n.e. not expressed. (**B**) Analysis of Ebf1 direct targets in a mesenchymal cell line. Binding is represented as a percentage of input chromatin, and error bars represent standard deviation of duplicate ChIP experiments.

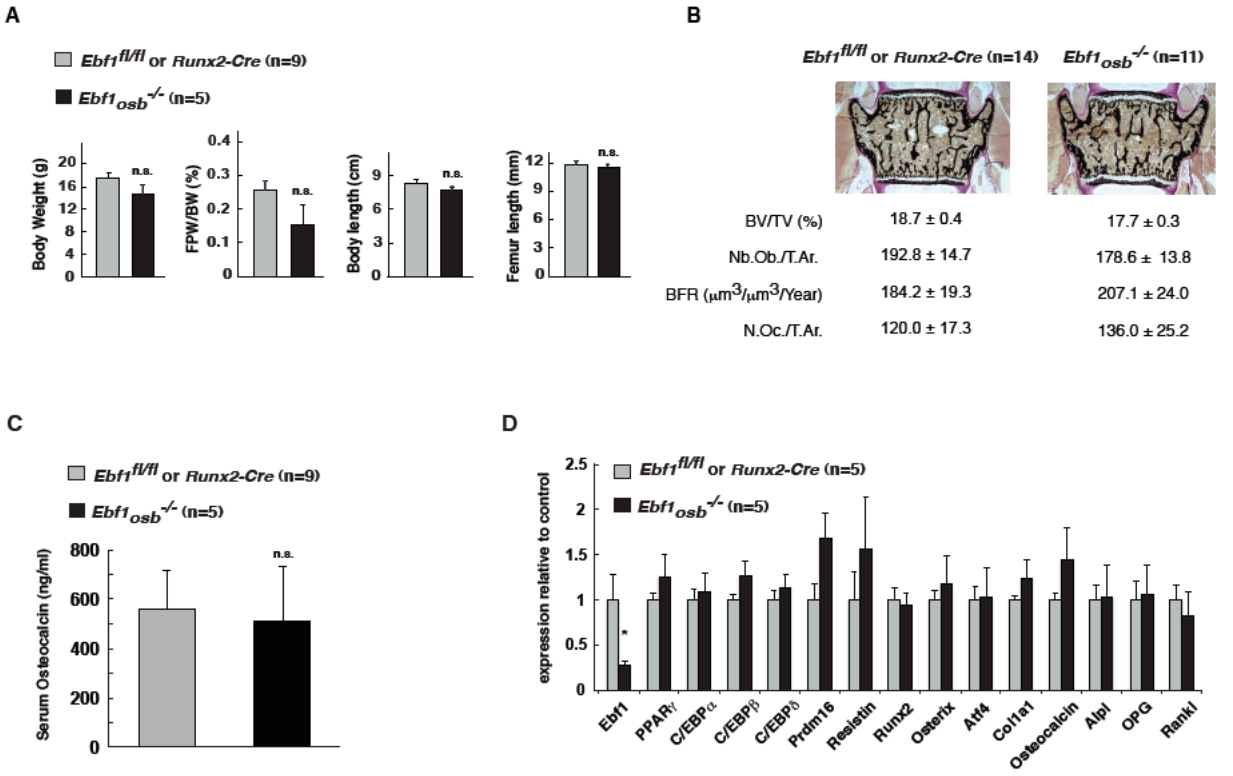


Figure 2-3. Phenotype analysis of *Ebf1*$_{osb}^{-/-}$. (**A**) Body weight, percent gonadal fat (FPW/BW), body length, and femur length, (**B**) Bone histomorphetric analysis, (**C**) osteocalcin measurement in serum (**D**) qPCR analysis of isolated bone, and (**D**) adipocytes of *Ebf1*$_{osb}^{-/-}$ mice.

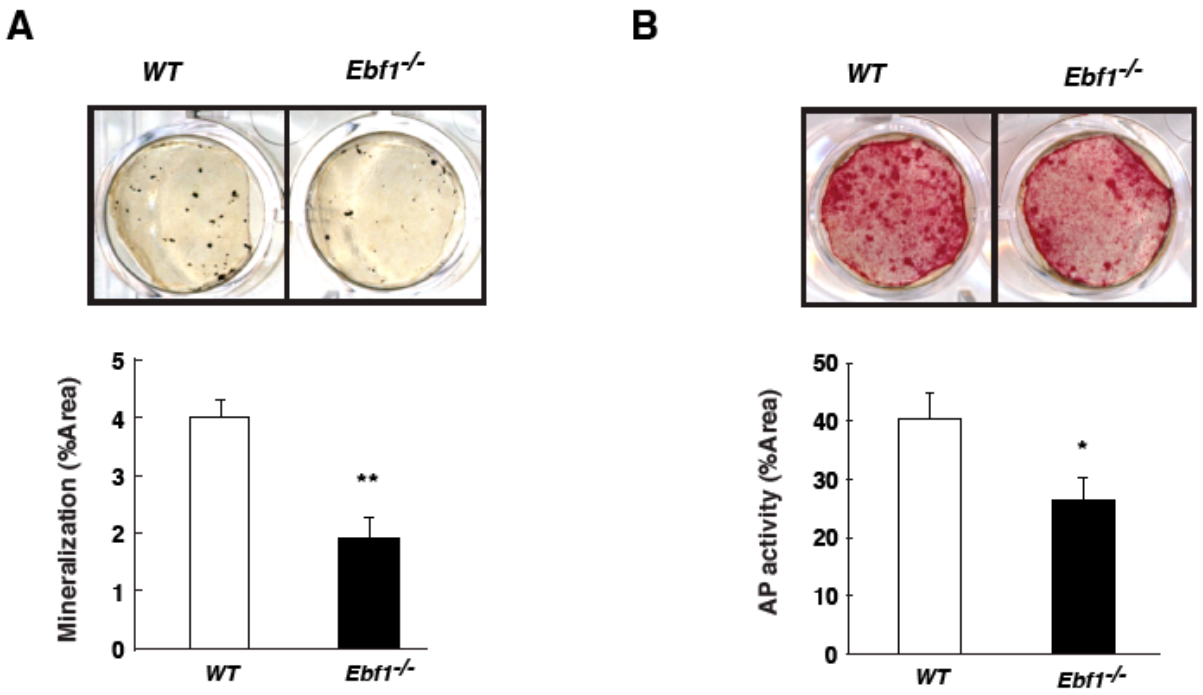
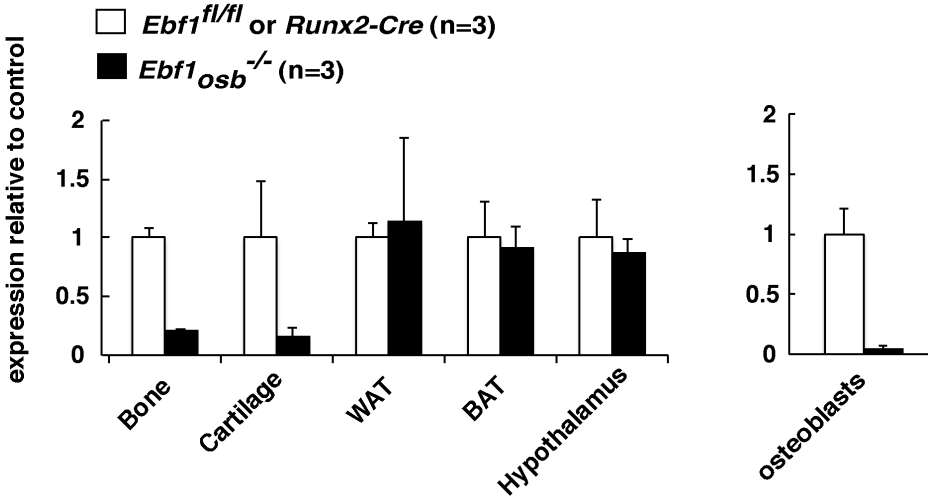


Figure 2-4. Differentiated primary osteoblasts persistently lacking *Ebf1* by (**A**) von Kossa, and (**B**) Alkaline phosphatase activity



Supplementary Figure 2-1. Deletion of the *Ebf1* allele in indicated tissues and in primary osteoblasts isolated from adult bone marrow.

Chapter Three: T-Cell Protein Tyrosine Phosphatase (TC-PTP) Regulates Bone Resorption and Whole-Body Insulin Sensitivity Through Its Expression in Osteoblasts

Tiffany Zee, Carmine Settembre, Robert L. Levine, and Gerard Karsenty. *Mol Cell Biol*. 32(6):1080-8 (2012)

Preface

The completion of this work is owed to the contribution of the people represented on the authorship list and with the generous help of others listed below. Dr. Carmine Settembre generated the *Ptpn1* and *Ptpn2* floxed allele mice. Dr. Robert Levine created the GST-PTPDA substrate-trapping mutant constructs. Dr. Tatsuya Yoshizawa assisted me with the glucose-stimulated insulin secretion test, and Dr. Mathieu Ferron for his generous help with coculture techniques. Dr. Gerard Karsenty conceived the study. Under the mentorship of Dr. Gerard Karsenty, I performed the rest of the work. This work was supported by the National Institutes of Health training grant Hormones: Biochemistry and Molecular Biology – T32DK07328 (T.Z.) and grant from the National Institutes of Health (G.K.)

Summary

Insulin signaling in osteoblasts contributes to whole body glucose homeostasis in the mouse and in humans by increasing the activity of osteocalcin. The osteoblast insulin signaling cascade is negatively regulated by ESP, a tyrosine phosphatase dephosphorylating the insulin receptor. *Esp* is one of many tyrosine phosphatases expressed in osteoblasts, and this observation suggests that other protein tyrosine phosphatases may contribute to the attenuation of insulin receptor phosphorylation in this cell type. In this study, we sought to identify additional PTP(s) that like ESP, would function in the osteoblast to regulate insulin signaling and thus affect activity of the insulin-sensitizing hormone osteocalcin. For that purpose, we used as criteria, expression in osteoblasts, regulation by isoproterenol, and ability to trap the insulin receptor in a substrate-trapping assay. Here we show that the T-cell protein tyrosine phosphatase (TC-PTP) regulates insulin receptor phosphorylation in the osteoblast, thus compromising bone resorption and bioactivity of osteocalcin. Accordingly, osteoblast-specific deletion of TC-PTP promotes insulin sensitivity in an osteocalcin-dependent manner. This study increases the number of genes involved in the bone regulation of glucose homeostasis.

Introduction

The tenuous cross-talk existing between bone remodeling and energy metabolism was first demonstrated *in vivo* through the realization that leptin, an adipocyte-derived hormone, inhibits both appetite (Flier and Elmquist, 1997; Friedman and Halaas, 1998; Spiegelman and Flier, 2001) and bone mass accrual (Ducy et al., 2000). The existence of this crosstalk was then further substantiated by the observation that in turn, osteoblasts regulate whole-body glucose metabolism, through secretion of the hormone osteocalcin that favors insulin secretion, insulin sensitivity, and increases energy expenditure (Ferron et al., 2008; Lee et al., 2007; Rached et al., 2010a).

Like other peptide hormones, osteocalcin undergoes significant post-translational modifications before being released into the general circulation (Hauschka et al., 1989b; Steiner, 2011). Specifically, osteocalcin, which is secreted by osteoblasts as a γ-carboxylated protein, must be decarboxylated to become activated and able to fulfill its endocrine functions (Lee et al., 2007). This activation of osteocalcin has been shown to occur outside the osteoblast, in the bone resorption lacunae (Ferron et al., 2010a). As the only mechanism known to decarboxylate proteins outside of the cell is by incubating them in an acidic pH, the passage of osteocalcin through the acidic microenvironment of the resorption lacunae allows it to become decarboxylated and thus activated (Engelke et al., 1991; Ferron et al., 2010a). In effect, the resorbing function of osteoclasts favors glucose homeostasis by activating osteocalcin (Ferron et al., 2010a).

In addition to being an endocrine cell, the osteoblast receives many endocrine signals, one of them being insulin. Among other functions, insulin signaling in osteoblast inhibits the expression of *Opg*, a gene encoding a decoy receptor for the RANKL osteoclast differentiation

factor. As a consequence, insulin signals to the osteoblast to promote bone resorption, osteocalcin bioactivity, and thereby its own secretion (Ferron et al., 2010a).

As it is the case in other insulin sensitive cells such as in the hepatocytes and myocytes, the insulin signaling cascade in the osteoblast is tightly regulated (Saltiel and Kahn, 2001; Schlessinger, 2000; Tonks, 2006). In particular, protein tyrosine phosphatases (PTPs) play a crucial role in attenuating insulin receptor phosphorylation to limit insulin signaling in many cell types and maintain glucose homeostasis (Hunter, 1995; Schlessinger, 2000; Tonks, 2006). To date, the only tyrosine phosphatase expressed in the mouse osteoblast that has been shown to dephosphorylate the insulin receptor is ESP (Ferron et al., 2010a). As a result, the deletion of *Esp* specifically in the osteoblast enhances insulin signaling, increases circulating levels of active osteocalcin, and accordingly, favors glucose tolerance, insulin sensitivity, and energy expenditure (Ferron et al., 2010a; Lee et al., 2007). This and other experiments have identified ESP as a major intracellular regulator of osteocalcin's endocrine function in the mouse.

However, ESP is not the only PTP present in osteoblasts, an observation suggesting that other PTPs in addition to ESP might contribute to the regulation of glucose metabolism through their expression in osteoblasts. If this were the case, it would strengthen the notion that bone is involved in the regulation of glucose metabolism.

To address this question, and since ESP belongs to the family of classical PTPS, which are defined by their specificity for phosphotyrosine (Alonso et al., 2004; Barr et al., 2009), we tested all 37 other mammalian classical PTPs for their ability to bind to the endogenous insulin receptor in osteoblasts and to be up-regulated by isoproterenol, as is *Esp* (Hinoi et al., 2008). Only one PTP was able to bind to the osteoblast insulin receptor and respond to isoproterenol treatment – TC-PTP. We show here that TC-PTP regulates osteocalcin bioactivity by inhibiting

bone resorption, thus affecting whole-body glucose metabolism. Hence, these results identify a role for TC-PTP as a novel regulator of energy metabolism through its expression in the osteoblast.

Methods

Animal studies

All mice studied were age-matched littermate males on a mixed (87.5% C57BL/6J; 12.5% 129/Sv) background. Genotyping was performed by PCR using DNA extracted from tail tips; primer sequences are available upon request. All mice were maintained on a 12-hour light/dark cycle in a barrier facility with free access to standard chow and water, and analyzed at 5-7 weeks of age.

Metabolic Measurements

Glucose in tail blood was measured using a glucometer (Accucheck). For the glucose tolerance test (GTT), mice were fasted overnight and injected with 2 kg D-glucose per kg body weight. Blood glucose was assayed immediately before and at 15, 30, 60, and 120 minutes post-injection. For the insulin tolerance test (ITT), mice were fasted for 4 hours and injected with 0.5 U/kg insulin (Humulin R, Lilly). Blood glucose was measured immediately before and at 30, 60, 90, and 120 minutes post-injection. For the glucose stimulated insulin secretion (GSIS), mice were fasted overnight and injected with 2 kg D-glucose per kg body weight. Tail blood was collected immediately before and at 2, 5, 15, and 30 minutes post-injection (Lee et al., 2007). ELISA were used to determine serum insulin (Mercodia), serum CTx (Serum Crosslaps, IDS),

and serum GLU, GLA, and total osteocalcin as previously described (Ferron et al., 2010b). Whole calvaria was collected in mice injected through the inferior vena cava after overnight fasting. Quantification of western blot was performed using ImageJ.

Substrate trapping and Coimmunoprecipitation

GST-PTPDA proteins were generated by cloning the non-receptor PTPs and cytoplasmic phosphatase domains of the receptor PTPs into the BamHI site of pGEX 4T3. Site directed mutagenesis was used to mutate the catalytic aspartate acid residue to inactive alanine, as previously described (Flint et al., 1997b). The expression vectors were then transformed into BL21 (DE3) pLysS bacteria (Novagen). Recombinant GST-PTPDA proteins were induced and purified using glutathione-sepharose beads and then immediately incubated in lysate of pervanadate treated ROS17/2.8 cells, as previously described (Ferron et al., 2010a). The proteins were resolved on SDS-PAGE followed by western blot. Anti-IRβ and anti-pTyr were obtained from Cell Signaling Technology. For coimmunoprecipitation assays, PTP1B and TC-PTP and their respective substrate trapping mutants were cloned into the EcoRI and BamHI site of the pFLAG-5a expression vector. ROS17/2.8 cells were transfected with FLAG-fusion protein or empty vector and InsR expressed in pCNDA3.1 using Lipofectamine 2000. 24 hours post-transfection, FLAG-fusion protein were immunoprecipitated overnight and eluted as previously described (Ferron et al., 2010a) and resolved on SDS-PAGE followed by western blot. Anti-FLAG M2 affinity gel and 3X FLAG peptide was obtained from Sigma.

Cell Culture

Mouse primary osteoblasts were isolated as previously described (Ducy et al., 1997). siRNA knockdown was achieved by transfection with siRNA pools (On-target, Dharmacon) according to manufacturer instructions. Anti-Y1150/Y1151 IRβ antibody was obtained from Cell Signaling Technology. *Ptpn2*$^{+/+}$ or *Ptpn2*$^{-/-}$ osteoblasts were generated by infecting *Ptpn2*$^{flox/flox}$ osteoblasts with either GFP- or Cre-expressing adenovirus (University of Iowa). For the stimulation with isoproterenol and analysis of mineralized osteoblasts, cells were differentiated 3 days post-confluence in α-MEM medium containing 10% FBS, 10mM β-glycerophosphate, and 100μg/mL ascorbic acid. Osteoblasts differentiated for 5 days were stimulated with isoproterenol for 4 hours. Osteoblast/osteoclast cocultures were prepared as previously described (Ferron et al., 2010a). In vitro resorption activity of osteoclasts was measured using BD Biocoat Osteologic Bone Cell Culture System according to manufacturer's instructions. Primary osteoblasts mineralization was visualized using Von Kossa staining and quantified by ImageJ.

Gene Expression Analysis

RNA isolation, cDNA preparation, and qPCR analysis was performed using standard protocols and relative Ct values are standardized to Ct values of control β-actin or S18 (for osteoclasts). Exonic qPCR primers used to compare expression were normalized on a standard curve of mouse genomic DNA.

Bone Histomorphometry

Static and dynamic histomorphometric analyses were performed on vertebral column specimens collected from 7-week old mice using undecalcified sections according to standard protocols using the Osteomeasure analysis system (Osteometrics).

Statistics

Results are given as means ± standard errors of the mean. Statistical analyses were performed using unpaired, two-tailed Student's t-test. For all experiments, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Results

TC-PTP and PTP1B bind to the insulin receptor in osteoblasts

With the goal of identifying additional protein tyrosine phosphatases that could dephosphorylate the insulin receptor in osteoblasts, we tested members of the classical protein tyrosine phosphatase (PTP) family for *in vitro* binding of the insulin receptor and expression in osteoblasts.

For the first purpose, we generated substrate-trapping mutants of all PTPs by introducing a DA substitution mutation in each phosphatase domain that abolishes catalytic activity but preserves the ability of the PTPs to bind substrate(s) (Blanchetot et al., 2005; Flint et al., 1997b). These mutant GST-fusion proteins were incubated with extracts from pervanadate-treated ROS17/2.8 osteoblast cells and then pulled down using glutathione beads. As shown in Figure 3-1A, Western blot analysis indicated that, in addition to ESP, five PTPs were able to interact

directly with the endogenous β-subunit of the insulin receptor in osteoblasts: PTP1B (*Ptpn1*), TC-PTP (*Ptpn2*), PTPCL (*Ptpn3*), PTPMEG (*Ptpn4*), and MEG2 (*Ptpn9*).

In an effort to further narrow down our search, we relied on one particularity of the ESP/osteocalcin metabolic pathway. *Esp* gene expression in osteoblasts is upregulated upon treatment of osteoblasts with the β2-adrenergic receptor agonist isoproterenol (Hinoi et al., 2008). We considered this response a defining feature of the osteocalcin regulation of energy metabolism, and thus tested whether expression of *Ptpn1, Ptpn2, Ptpn3, Ptpn4,* and *Ptpn9* was affected by isoproterenol treatment of osteoblasts. Remarkably, *Ptpn2* expression was significantly affected by isoproterenol treatment in mineralized osteoblasts (Fig. 3-1B). These results identify TC-PTP as the leading candidate to be a second PTP involved in the osteoblast regulation of energy metabolism.

To formally demonstrate that the insulin receptor is a physiological substrate of TC-PTP in osteoblasts, we performed coimmunoprecipitation with the TC-PTP substrate trapping mutants expressed in the osteoblast, using PTP1B as a positive control in this assay (Flint et al., 1997b). For that purpose, we transfected ROS17/2.8 osteoblast cells with flag-tagged wild-type and substrate-trapping mutants of PTP1B, TC-PTP or ESP. IRβ coimmunoprecipitated with DA-substrate trapping mutants of PTP1B, TC-PTP, and ESP while it did not with FLAG alone (Fig. 3-1C). Interestingly, wild-type ESP protein also exhibited binding to the insulin receptor, which was not detected with PTP1B and TC-PTP, suggesting that ESP can bind to the insulin receptor independently of its phosphatase domain (Fig. 3-1C). These results confirm that the insulin receptor is a substrate of PTP1B and TC-PTP in rodent osteoblasts.

TC-PTP is expressed in insulin-responsive tissues, including osteoblasts

In view of these results we then determined the level of expression of TC-PTP in bone cells *in vivo*. Western blot and qPCR analysis confirmed the presence of mRNAs encoding TC-PTP (*Ptpn2*) in isolated femur tissue, coinciding with the presence of the insulin receptor (Fig. 3-1D, 3-1E). When comparing the relative levels of *Ptpn1*, *Ptpn2*, and *Esp* expression by qPCR analysis using exonic primers normalized to genomic DNA, we observed that in all tissues tested, *Ptpn2* expression exceeded that of both *Esp* and *Ptpn1*, with prominent expression in bone (Fig. 3-1D). Because bone is composed of multiple cells types, we also analyzed expression of the PTPs in primary osteoblasts isolated from newborn calvaria. Consistent with data obtained from whole bone, we observed that *Ptpn2* was more highly expressed in primary osteoblasts than *Ptpn1* and *Esp* (Fig. 3-1F). Interestingly, *Ptpn2* but not *Ptpn1* expression was enhanced when osteoblasts were induced to fully differentiate in medium containing β-glycerophosphate and ascorbic acid (Fig. 3-1F).

Generation of mice lacking TC-PTP or PTP1B specifically in the osteoblast

To study the function of TC-PTP in osteoblasts *in vivo*, we used a cell-specific loss-of-function strategy in the mouse and generated a floxed allele of *Ptpn2* and *Ptpn1* by homologous recombination in mouse ES cells. For the *Ptpn2* floxed allele targeting strategy, we used a targeting vector including the genomic sequence from exon 4 to exon 8. Two *loxP* sites were added to flank exons 5 to 7, which contain the catalytic domain of TC-PTP (Fig. 3-2A). In addition, a neomycin (NeoR) cassette flanked by two *Frt* sites was inserted in the intron between exon 6 and 7 (Fig. 3-2A). Southern blot analysis identified the targeted *Ptpn2* allele using a 5' probe directed to intron 2 and a 3' probe directed to the region containing exon 9 and a portion of

intron 8 (Fig. 3-2A). The inserted NeoR cassette was subsequently removed by crossing mice containing the targeted allele with mice expressing Flp recombinase. NeoR excision was verified by PCR analysis (Fig. 3-2A).

To delete TC-PTP specifically in osteoblasts ($Ptpn2_{osb}^{-/-}$ mice) mice harboring the floxed allele of *Ptpn2* were crossed with mice expressing the *α1(I)collagen-Cre* transgene (Dacquin et al., 2002). PCR analysis confirmed that recombination of the *Ptpn2* floxed allele was limited to bone only (Fig. 3-2B). $Ptpn2_{osb}^{-/-}$ mice were born at the expected Mendelian ratio and appeared phenotypically normal at birth, indicating that *Ptpn2* expression in osteoblasts is dispensable for normal embryonic development.

To generate the *Ptpn1* floxed allele, we used a targeting vector including the genomic sequence from exon 3 to exon 9, similarly to what has been done previously (Bence et al., 2006a). Two *loxP* sites were added to flank exons 6 to 8, since the catalytic domain of PTP1B is contained in exons 7 and 8 (Fig. 3-2A). A neomycin (NeoR) cassette flanked by two *Frt* sites was inserted in the intron between exon 8 and 9 (Fig. 3-2A). Southern blot analysis identified the targeted *Ptpn1* allele using a 5' probe directed to intron 2 and a 3' probe directed to the region containing exon 9 (Fig. 3-2A). The inserted NeoR cassette was subsequently removed by crossing mice containing the targeted allele with mice expressing Flp recombinase and confirmed by PCR analysis (Fig. 3-2B). qPCR analysis of bone marrow derived osteoblasts estimated recombination efficiency of 47.3% (Fig. 3-2C).

Similarly to $Ptpn2_{osb}^{-/-}$, mice harboring the floxed allele of *Ptpn1* were crossed with mice expressing the *α1(I)collagen-Cre* transgene (Fig, 3-2D, (Dacquin et al., 2002). PCR and qPCR analysis confirmed that recombination of the *Ptpn2* floxed allele was limited to bone only and at

approximately 53.6% efficiency (Fig. 3-2E, 3-2F). *Ptpn1*$_{osb}^{-/-}$ mice were also born at the expected Mendelian ratio and appeared phenotypically normal throughout life.

TC-PTP regulates insulin signaling in osteoblasts

Next we evaluated the contribution of TC-PTP in the regulation of insulin signaling in isolated osteoblasts, and compared it to that of PTP1B. Primary cultures of calvarial osteoblasts were transfected with siRNA to suppress expression of either PTP1B or TC-PTP. In response to insulin stimulation, phosphorylation of the insulin receptor on the Y1150 and Y1151 residues was enhanced in osteoblasts deficient specifically for TC-PTP (Fig. 3-3A). There was no apparent increase in phosphorylation of the insulin receptor phosphorylation in osteoblasts lacking PTP1B. However, normalization of the signal with the amount of total insulin receptor indicated that phosphorylation was in fact, increased nearly 2-fold in these osteoblasts, compared to the 5-fold increase observed in osteoblasts treated with TC-PTP siRNA(Fig. 3-3A). We also observed that osteoblasts lacking either PTP1B or TC-PTP had increased basal expression of the insulin target gene *Gsy1*, though this increase reached statistical significance only in TC-PTP-deficient osteoblasts (Fig. 3-3B). Taken together, these results suggested that TC-PTP might play a more important role in regulating insulin signaling in the mouse osteoblast.

In view of these results, we focused our subsequent work on TC-PTP and asked whether insulin signaling is enhanced in the osteoblasts of *Ptpn2*$_{osb}^{-/-}$ mice. Western blot analysis of whole calvaria isolated after intravenous injection of 0.5 U/kg insulin revealed that insulin receptor phosphorylation was increased in *Ptpn2*$_{osb}^{-/-}$ calvaria (Fig. 3-3C). It is important to underline that because bone is a mixture of multiple cell types and *Ptpn2* deletion is specific to osteoblasts only, the difference in insulin receptor phosphorylation in whole calvaria was

expected to be subtle. Nevertheless, phosphorylation of the insulin receptor on Y1150 and Y1151 was enhanced more than 30% in the calvaria of *Ptpn2*$_{osb}^{-/-}$ mice compared to their floxed littermate controls, confirming that TC-PTP regulates insulin receptor phosphorylation *in vivo* (Fig. 3-3C).

Mice lacking TC-PTP specifically in osteoblasts demonstrate increased osteocalcin bioactivity and insulin sensitivity

To test if TC-PTP or PTP1B regulates osteocalcin bioactivity, we quantified, using an ELISA developed in the laboratory (Ferron et al., 2010b), serum undercarboxylated osteocalcin (GLU13-OCN) levels in *Ptpn2*$_{osb}^{-/-}$ and *Ptpn1*$_{osb}^{-/-}$ mice. Compared to their WT littermates, *Ptpn2*$_{osb}^{-/-}$ mice have increased serum levels of GLU-13 OCN, though osteocalcin bioactivity was not significantly altered in *Ptpn1*$_{osb}^{-/-}$ mice (Fig. 3-4A, 3-4B). This result confirmed that the increase in insulin signaling in the osteoblasts lacking TC-PTP coincided with an increase in serum undercarboxylated, i.e., active osteocalcin (Fig. 3-4A). This finding suggested that one function of TC-PTP in osteoblasts is to regulate osteocalcin bioactivity.

Given the influence exerted by osteocalcin on glucose metabolism (Lee et al., 2007), we then asked whether osteoblast-specific deletion of TC-PTP might affect whole-body glucose metabolism. A glucose tolerance test (GTT) performed at 6 weeks of age revealed no difference in glucose tolerance in *Ptpn2*$_{osb}^{-/-}$ mice (Fig. 3-4C). As expected, there was similarly no difference in glucose tolerance of *Ptpn1*$_{osb}^{-/-}$ mice (Fig. 3-4D). However, *Ptpn2*$_{osb}^{-/-}$ mice consistently exhibited increased insulin sensitivity as measured by an insulin tolerance test (ITT) (Fig. 3-4E). This effect was not observed in *Ptpn1*$_{osb}^{-/-}$ mice (Fig. 3-4F). The increase in insulin sensitivity of *Ptpn2*$_{osb}^{-/-}$ mice was also apparent when calculated as the area under the curve of

the insulin tolerance test (Fig. 3-4G). To test whether glucose homeostasis was altered in the insulin-sensitive *Ptpn2*$_{osb}^{-/-}$ mice under challenged conditions, we performed a glucose tolerance test on mice that were fed a high fat diet for 5 weeks. Glucose tolerance was also unaffected under these conditions (Fig. 3-4I). These data indicated that osteoblast expression of TC-PTP but not of PTP1B, regulates insulin sensitivity, presumably by influencing osteocalcin bioactivity. A glucose stimulated insulin secretion test (GSIS) confirmed that insulin secretion was normal in *Ptpn2*$_{osb}^{-/-}$ mice (Fig. 3-4H). Energy expenditure was also increased in *Ptpn2*$_{osb}^{-/-}$ mice during the day cycle (Fig. 3-4J). These results indicated that although the increase in activated osteocalcin of *Ptpn2*$_{osb}^{-/-}$ mice was sufficient to affect insulin sensitivity and energy expenditure, it was insufficient to affect insulin secretion, glucose tolerance, and night cycle energy expenditure. The role of TC-PTP is not identical to that of ESP, as *Esp*$_{osb}^{-/-}$ mice suffer from both hypoglycemia and hyperinsulinemia and display increased energy expenditure in both day and night cycles (Lee et al., 2007).

TC-PTP affects bone resorption through its expression in osteoblasts

We had previously shown that insulin signaling in osteoblasts is a molecular determinant of osteocalcin bioactivity by promoting bone resorption. Accordingly, bone resorption is higher in *Esp*$_{osb}^{-/-}$ mice that are a model of a gain of function of insulin signaling in osteoblasts and have an increase in active osteocalcin (Ferron et al., 2010a). To determine whether TC-PTP regulates osteocalcin activity by utilizing the same mechanism of action, we asked whether TC-PTP influences bone resorption.

For that purpose we performed a classical co-culture assay (Takahashi et al., 1988). In this experiment, wild-type (WT) or *Ptpn2*$^{-/-}$ osteoblasts were cultured with WT monocytes in the

presence of $VitD_3$ and PGE_2 for 8 days. At the end of the experiment, we stained for tartrate resistant acid phosphatase (TRAP), an enzyme expressed only in mature osteoclasts, to evaluate osteoclast differentiation (Burstone, 1959). TRAP staining revealed that osteoblasts lacking TC-PTP (*Ptpn2*$^{-/-}$) induced greater osteoclast differentiation than WT osteoblasts, as quantified by number of TRAP-positive cells (Fig. 3-5A). Functional analysis confirmed that resorptive function of osteoclasts was also increased, as the resorptive pit area covered by osteoclasts co-cultured with *Ptpn2*$^{-/-}$ osteoblasts was significantly larger than that of osteoclasts cultured with WT osteoblasts (Fig. 3-5B). Osteoblasts lacking TC-PTP (*Ptpn2*$^{-/-}$) demonstrated decreased expression of *Opg* but no change in *Rankl* expression, as do *Esp*$^{-/-}$ osteoblasts (Fig. 3-5C) (Ferron et al., 2010a). In addition, we observed an increase in expression of *Ccl8*, a monocyte chemoattractant supporting osteoclast formation (Fig. 3-5C) (Winslow et al., 2006). These results confirmed that TC-PTP, primarily through its regulation of insulin signaling in osteoblasts, influences bone resorption. Consistent with this contention, expression of *Tcirg1*, a gene expressed in osteoclasts but whose expression is regulated by insulin signaling in osteoblasts (Ferron et al., 2010a), was significantly increased in the osteoclasts cocultured with *Ptpn2*$^{-/-}$ osteoblasts (Fig. 3-5D). The same was true for *Clcn7*, a chloride channel also regulating the acidity of the resorption lacunae (27, (Schaller et al., 2005), and *Trap*, a marker of osteoclasts (Andersson and Marks, 1989) (Fig. 3-5D). Taken together, these experiments indicate that in cell culture, TC-PTP, presumably through its ability to inactivate the insulin receptor in the osteoblasts, affects *Opg* expression and thus regulates osteoclast differentiation and function.

Accordingly, when compared to control littermates, *Ptpn2*$_{osb}^{-/-}$ mice have increased osteoclast activity as demonstrated by increased serum levels of CTx, a marker of bone resorption (Fig. 3-5E) (Rosen et al., 2000). However, osteoclast number in these mice was not

significantly changed, indicating that *in vivo* deletion of TC-PTP is sufficient to affect osteoclast activity but not osteoclast number (Fig 3-5F).

Bone histomorphometry analysis performed in vertebrae revealed that at 6 weeks of age, bone volume was slightly decreased, though this did not reach statistical significance (Fig. 3-5F). Osteoblast number and bone formation rate were also not significantly altered. Primary osteoblasts lacking *Ptpn2* similarly displayed no apparent proliferation and differentiation defect, as demonstrated by Van Kossa staining (Fig. 3-5F). This result suggests that TC-PTP does not affect osteoblast proliferation and ability to differentiate.

These data indicate that TC-PTP, by modulating insulin signaling in osteoblasts, is a determinant of osteoclast activity. It is through this mechanism in which TC-PTP affects osteocalcin bioactivity, and thus insulin sensitivity.

Discussion

We demonstrate here that in addition to ESP, another tyrosine phosphatase TC-PTP, regulates whole-body insulin sensitivity and day cycle energy expenditure by increasing osteocalcin activity through its expression in the osteoblast. These results further our understanding of the complexity of the regulation of osteocalcin activity and underscore the importance of the osteoblast as an endocrine cell type.

Using substrate-trapping assays and loss-of-function models, we show that a function of TC-PTP is to attenuate insulin signaling in the osteoblast. In view of these results, we generated mice lacking TC-PTP specifically in the osteoblast ($Ptpn2_{osb}^{-/-}$) and observed that the level of circulating active osteocalcin is increased in these mice compared to their littermate controls. This corresponded to an increase in whole-body insulin sensitivity and day cycle energy

expenditure in $Ptpn2_{osb}^{-/-}$ mice on a standard chow diet. These mice are therefore, a partial phenocopy of $Esp_{osb}^{-/-}$ mice, which, in addition, display an increase in insulin secretion and increase in energy expenditure in the night cycle. These results suggest that activated osteocalcin may differentially regulate insulin secretion and insulin sensitivity. Accordingly, target tissues may respond to different activation thresholds of osteocalcin.

Though its role in the mouse osteoblast is more robust, ESP is non-functional in humans (Cousin et al., 2004a). In contrast, TC-PTP is conserved in humans (*PTPN2*) and has been implicated as a susceptibility gene in early-onset Type I diabetes (Espino-Paisan et al., 2011). Though TC-PTP may have a more minor role in mouse osteoblasts, its function to regulate osteocalcin activity is translatable to humans. Further study will be required to investigate whether ESP and TC-PTP regulate insulin signaling cooperatively in the mouse osteoblast.

It thus remains to be seen if additional regulatory factors in the osteoblast are involved in the control of energy metabolism by bone. That deletion of *Ptpn2* in osteoblasts is insufficient to affect differentiation and proliferation is highly suggestive of the presence of other factors affecting insulin receptor signaling in osteoblasts (Fulzele et al., 2010). In particular, the role of the other tyrosine phosphatases expressed in osteoblasts that can bind the insulin receptor, but that are not regulated by isoproterenol stimulation, will need to be investigated. That *Ptpn2* expression is stimulated upon treatment of osteoblasts with isoproterenol also raises the question of whether osteoblast expression of *Ptpn2* contributes to the sympathetic regulation of bone mass and/or glucose homeostasis (Hinoi et al., 2008; Kajimura et al., 2011).

As insulin signaling in osteoblasts has been shown to favor bone resorption (Ferron et al., 2010a), we also examined how TC-PTP deficiency in these cells affects osteoclast function. A coculture system demonstrated that TC-PTP regulates the differentiation and resorptive activity

of osteoclasts though *Opg* expression in osteoblasts. Previous work had demonstrated a cell-autonomous function of TC-PTP in hematopoietic cell differentiation (Simoncic et al., 2006; Simoncic et al., 2002). Mice lacking TC-PTP globally exhibit an increase in osteoclast density and bone resorption (Doody et al., 2011). The current work expands the importance of TC-PTP by showing that it is able to regulate differentiation cell non-autonomously.

TC-PTP has been previously implicated in the regulation of metabolism, as demonstrated by its role in the liver and hypothalamus (Fukushima et al., 2010; Loh et al., 2011a). It also shares a phosphatase domain with that of PTP1B, the prototypical phosphatase of the insulin receptor and functional human homologue of ESP (Andersen et al., 2001; Ferron et al., 2010a). A ubiquitously expressed protein, PTP1B is implicated in the regulation of energy metabolism through its functions in muscle, fat, liver, and POMC-neurons of the brain (Agouni et al., 2011; Banno et al., 2010; Bence et al., 2006a; Delibegovic et al., 2007b; Delibegovic et al., 2009). Hence, we explored the possibility that PTP1B expression in the osteoblast may also contributes to the regulation of energy metabolism. *In vitro* experiments suggest that PTP1B does not seem to affect insulin receptor activation in osteoblasts, at least not to the same extent that TC-PTP does. Additionally, mice specifically lacking *Ptpn1* in osteoblasts ($Ptpn1_{osb}^{-/-}$) have no apparent differences in body weight, glucose tolerance, and insulin sensitivity compared to control littermates when fed on a normal diet.

That TC-PTP, but not PTP1B, regulates the osteocalcin activation pathway in mouse osteoblasts may be due to their relative levels of expression, as quantified by qPCR analysis (Fig. 1F). However, this difference may also be another example of divergent function between the two highly related phosphatases. Such a difference in the roles of PTP1B and TC-PTP has been documented for the regulation of cell spreading and adhesion (Stuible et al., 2008), pancreatic

islets response to ER stress (Bettaieb et al., 2011), as well as to the specificity of their substrates (Nievergall et al., 2010; Simoncic et al., 2006; Simoncic et al., 2002; Xu and Qu, 2008). In addition, PTP1B and TC-PTP have been shown to contribute differentially to glucose homeostasis through their expression in the muscle (Loh et al., 2011b). Evidence from previous studies has also indicated that at a molecular level, the difference in PTP1B and TC-PTP regulation of the insulin receptor can be dissected to the level of the phosphorylated residue (Galic et al., 2005).

This work extends our understanding of the bone regulation of energy metabolism, by identifying TC-PTP as an additional regulator of the osteocalcin-activation pathway. The finding that TC-PTP, a bona fide phosphatase of the insulin receptor, functions in osteoblasts to affect insulin sensitivity further establishes the role of insulin signaling in bone remodeling and energy metabolism.

Acknowledgements

This work was supported by the National Institutes of Health training grant Hormones: Biochemistry and Molecular Biology – T32DK07328 (T.Z.) and grant from the National Institutes of Health (G.K.).

We thank Dr. M. Ferron for reagents and help with coculture procedures, and Dr. T. Yoshizawa for assistance with GSIS.

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Figures

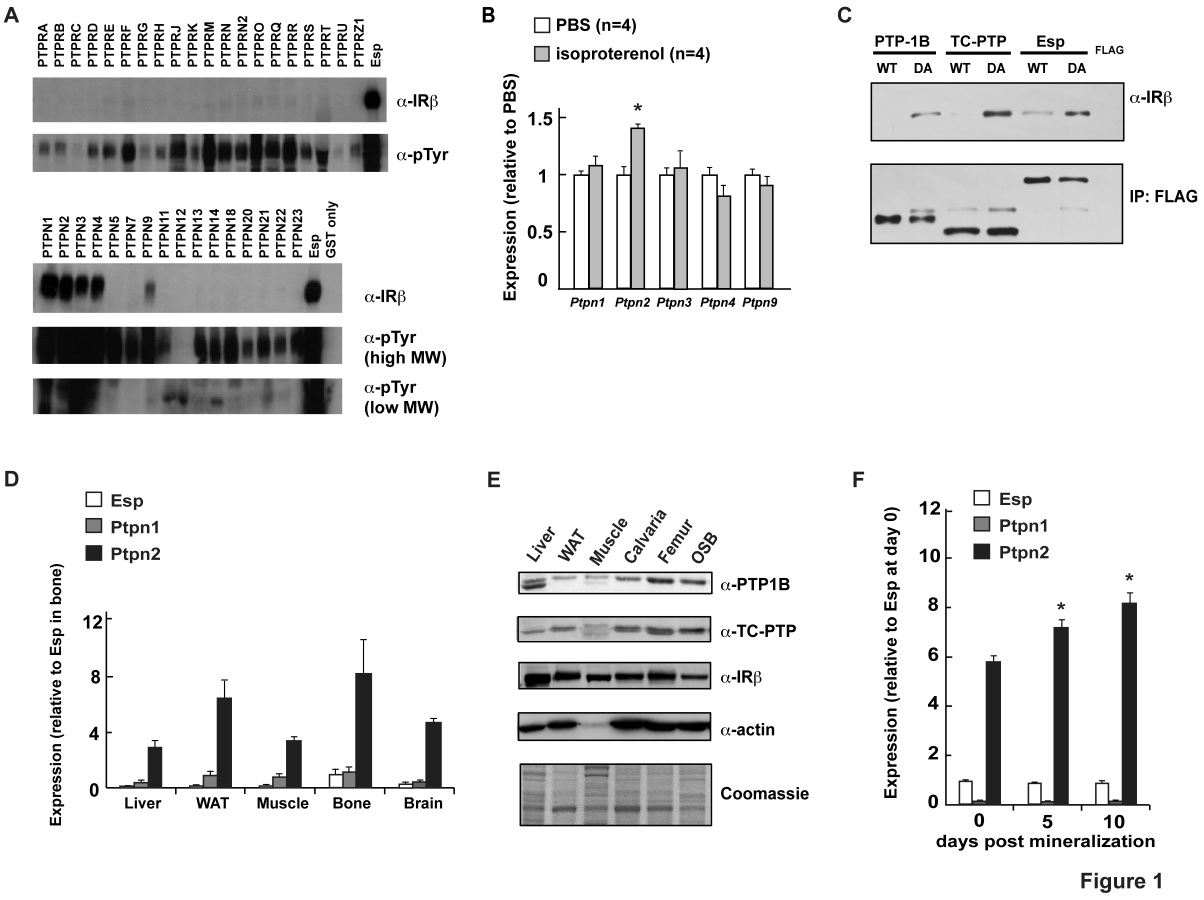

Figure 3-1. Identifying PTP(s) that parallel ESP.

(**A**) In vitro substrate trapping. Extracts from pervanadate-treated ROS17/2.8 cells were pulled down using GST only or DA mutants of PTP GST-fusion proteins. InsRβ was detected by western blot. An anti-phosphotyrosine (α-pTyr) was used for detection. (**B**) Stimulation of primary osteoblasts with isoproterenol (10μM). (**C**) In vivo substrate trapping. WT and DA FLAG tagged PTP1B and TC-PTP proteins were immunoprecipitated from ROS17/2.8 cells after 15 min stimulation with insulin (100 nM) (deleted). Immunoprecipitated proteins (IP) and total cell lysates were then analyzed by western blot. (**D**) qPCR expression analysis across different

tissues. (**E**) Expression analysis by western blot. (**F**) qPCR expression analysis in proliferative and differentiating mouse primary osteoblasts.

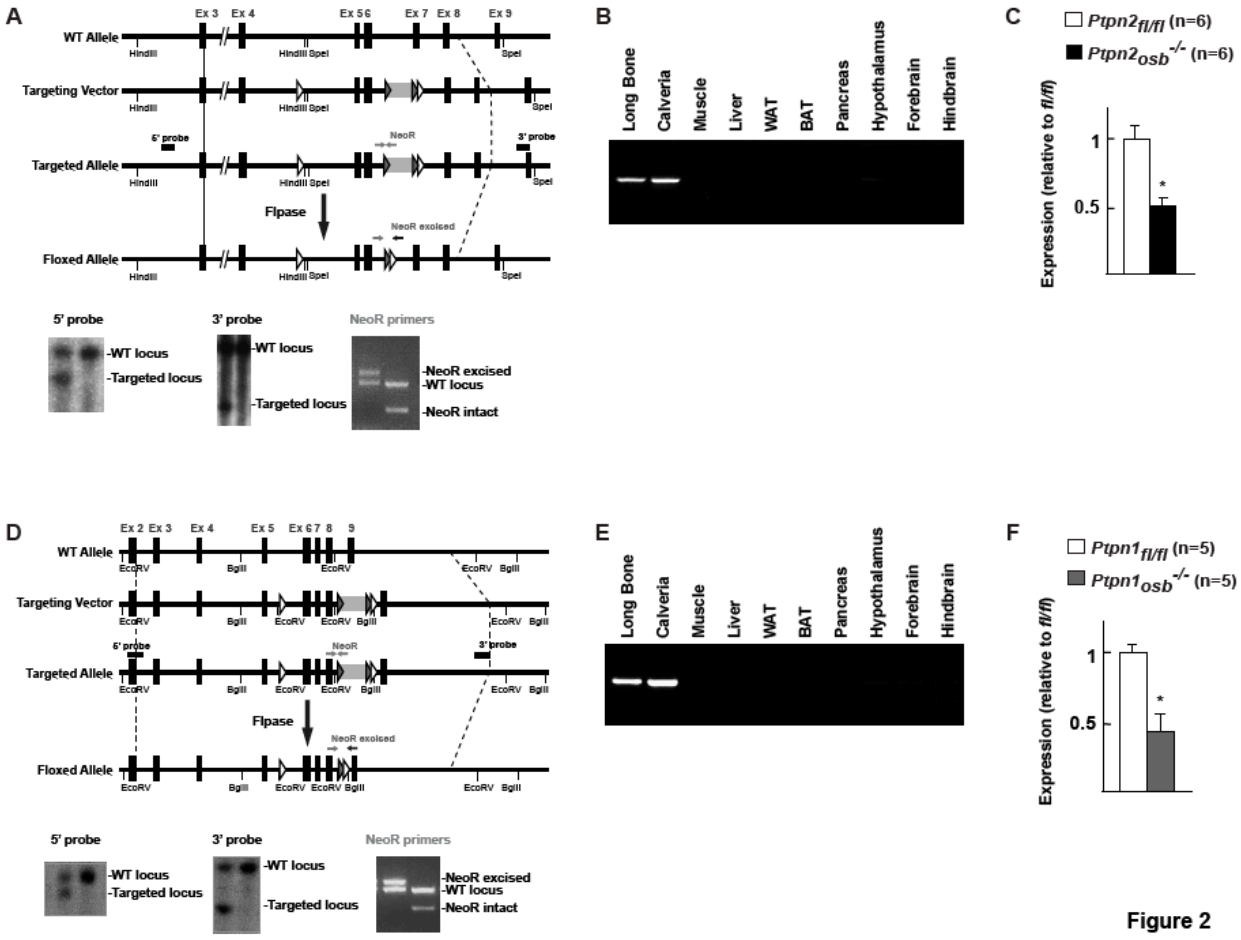

Figure 3-2. Generation of *Ptpn2*$_{osb}^{-/-}$ and *Ptpn1*$_{osb}^{-/-}$ mice.

(**A**) Generation of the *Ptpn2* floxed allele. (**B**) Deletion of the *Ptpn2* allele in indicated tissues. (**C**) qPCR analysis of *Ptpn2* expression in bone marrow derived osteoblasts (normalized to *osteocalcin* expression). (**D**) Generation of the *Ptpn1* floxed allele. (**E**) Deletion of the *Ptpn1* allele in indicated tissues. (**F**) qPCR analysis of *Ptpn1* expression in bone marrow derived osteoblasts (normalized to *osteocalcin* expression).

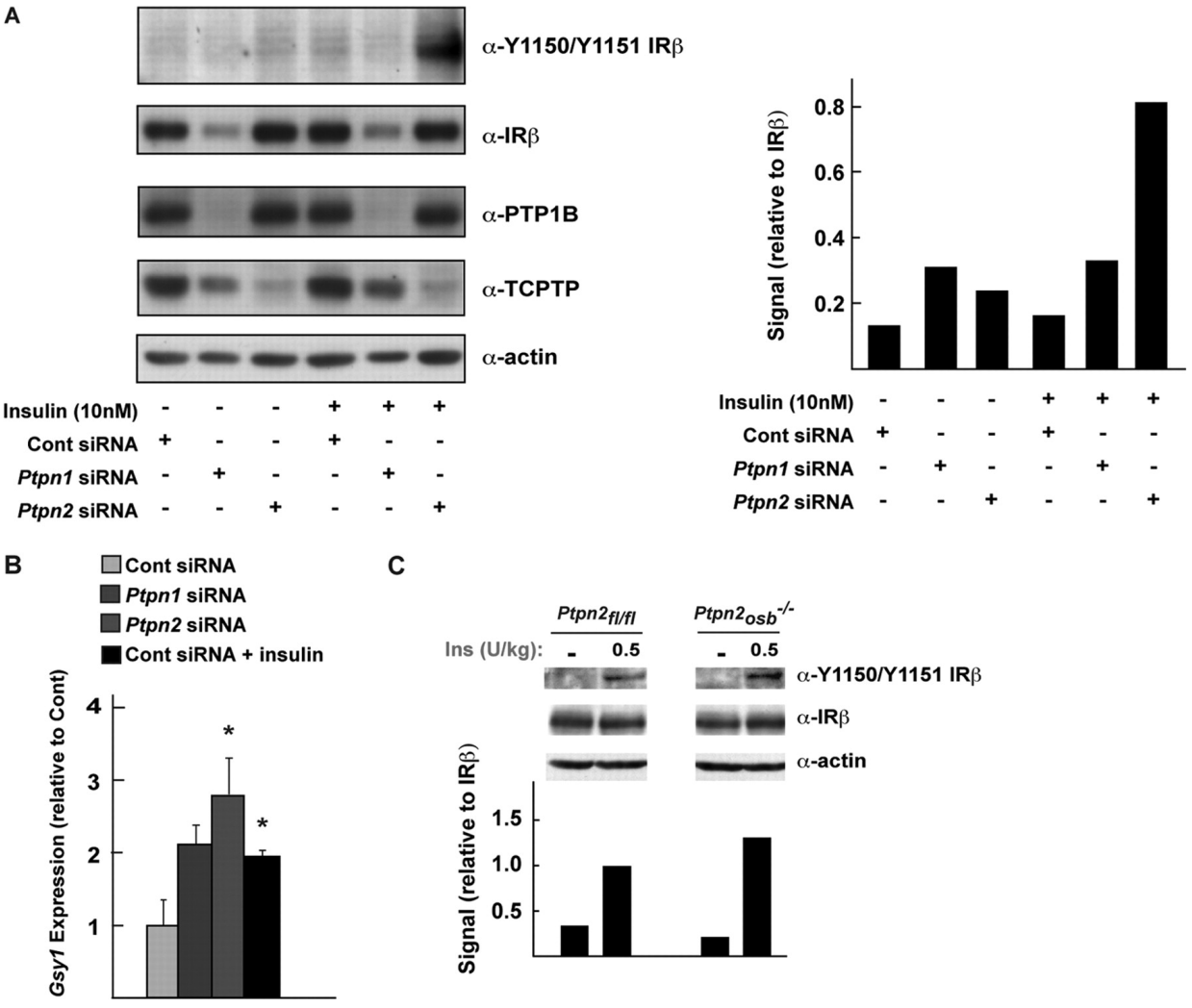

Figure 3-3. TC-PTP regulates insulin receptor phosphorylation in osteoblasts.

(**A**) Phosphorylation of the insulin receptor β-subunit 5 minutes post-treatment with insulin (10nM) in mouse primary osteoblasts. Quantification of signal normalized to IRβ using ImageJ. (**B**) Expression of the insulin target gene *Gsy1* in primary osteoblasts. Treatment of control is with 10nM insulin. (**C**) Phosphorylation of the insulin receptor β-subunit in whole calvaria after injection of insulin. Quantification of signal normalized to IRβ using ImageJ.

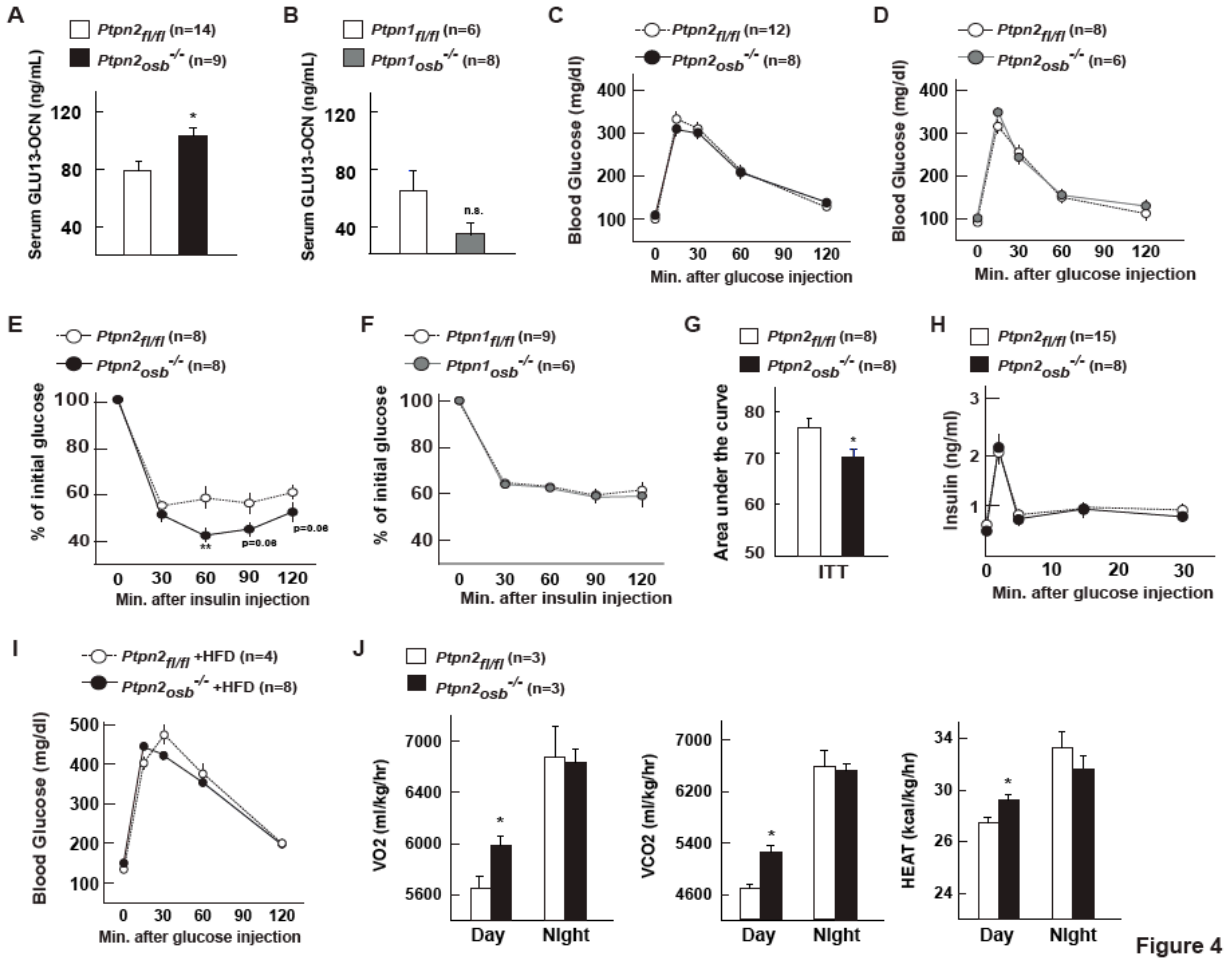

Figure 3-4. Improved insulin sensitivity in *Ptpn2$_{osb}^{-/-}$* mice.

(**A, B**) Total GLU13 osteocalcin levels in male (**A**) *Ptpn2$_{osb}^{-/-}$* and (**B**) *Ptpn1$_{osb}^{-/-}$* mice. (**C, D**) Glucose tolerance tests (GTTs) on male (**C**) *Ptpn2$_{osb}^{-/-}$* and (**D**) *Ptpn1 $_{osb}^{-/-}$* mice. (**E, F**) Insulin tolerance tests (ITTs) on male (**E**) *Ptpn2$_{osb}^{-/-}$* and (**F**) *Ptpn1 $_{osb}^{-/-}$* mice. (**G**) Area under the curve of (**E**). (**H**) Glucose stimulated insulin secretion test (GSIS) of *Ptpn2$_{osb}^{-/-}$* male mice. (**I**) GTT on male *Ptpn2$_{osb}^{-/-}$* mice on high fat diet. (**J**) Energy expenditure of *Ptpn2$_{osb}^{-/-}$* male mice.

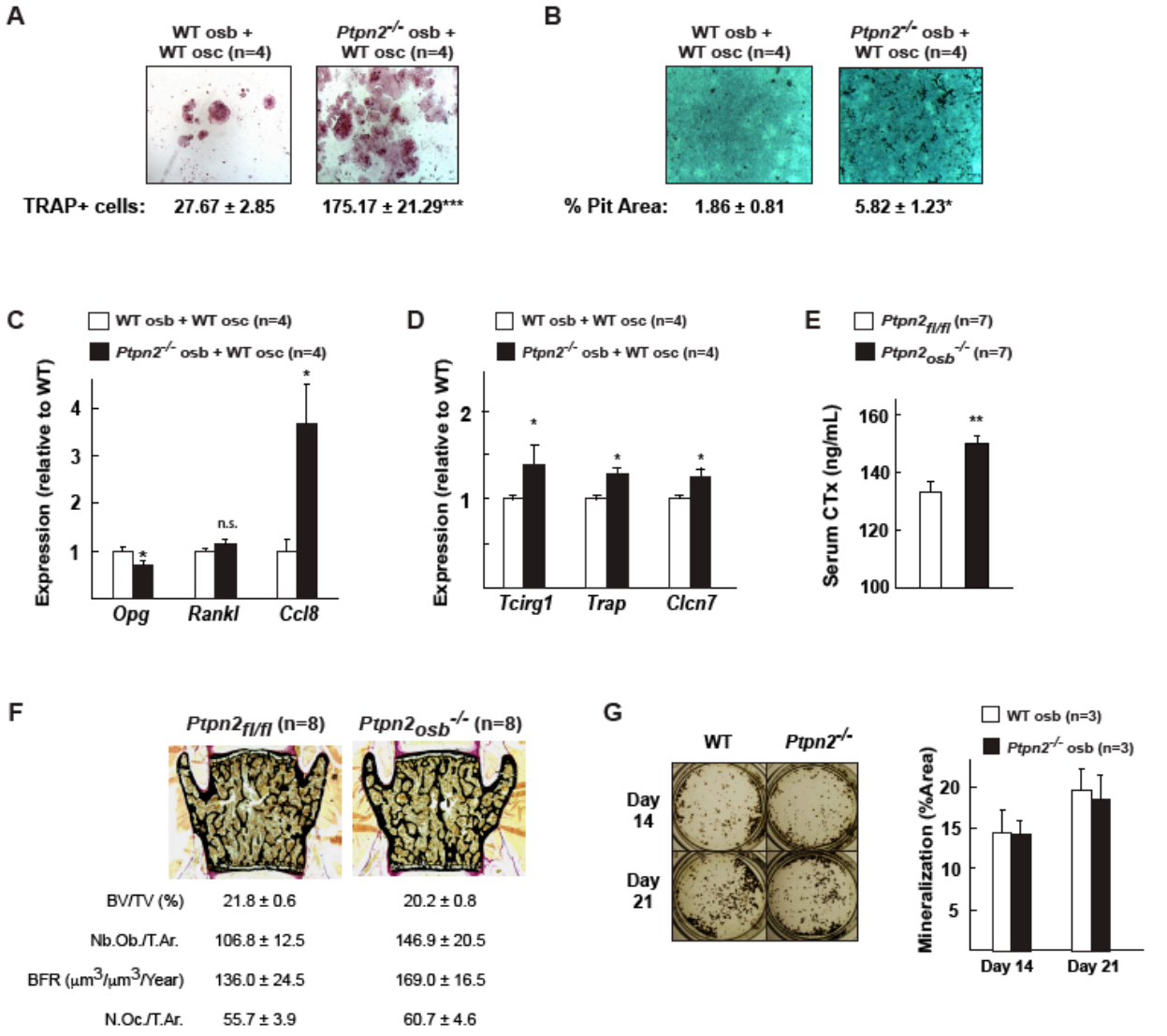

Figure 3-5. TC-PTP in osteoblasts regulates osteoclast differentiation.

(**A**) Representative pictures of TRAP staining of osteoclasts cocultured in the presence of WT or *Ptpn2*$^{-/-}$ osteoblasts. Quantification of the number of TRAP-positive cells. (**B**) Representative pictures of resorptive activity of osteoclasts cocultured in the presence of WT or *Ptpn2*$^{-/-}$ osteoblasts. Quantification of resorptive pit area. (**C**) qPCR analysis of *Opg*, *Rankl*, and *Ccl8* expression in WT or *Ptpn2*$^{-/-}$ osteoblasts cocultured with osteoclasts. (**D**) qPCR analysis of *Tcirg1*, *Trap*, and *Clcn7* expression in osteoclasts cocultured in presence of WT or *Ptpn2*$^{-/-}$ osteoblasts. (**E**) CTx serum levels in 6 week-old mice. (**F**) Representative pictures and

Histomorphometric analysis of *Ptpn2*$_{osb}^{-/-}$ mice and control *Ptpn2*$_{fl/fl}$ littermates. Bone Volume/Tissue Volume (BV/TV), Number of Osteoclasts/ Trabecular Area (N.Oc/T.Ar), Number of Osteoblasts/Trabecular Area (N.Ob/T.Ar), Bone Formation Rate/Bone Surface (BFR). (**G**) WT or *Ptpn2*$^{-/-}$ primary osteoblasts in culture differentiated for 14 and 21 days.

Chapter Four: Additional results and discussion

Ebf1 cell non-autonomous regulation of bone mass accrual

The finding that Ebf1 does not regulate osteoblast number cell-autonomously raises the following question: how does Ebf1 regulate bone mass accrual? There are many different tissues in which Ebf1 may be involved in regulating bone formation.

Previous studies have demonstrated that white adipose tissue, pancreas, and gut are all sources of hormones that affect bone formation. White adipose tissue is the source of the hormone leptin, which binds to its receptors in the brainstem to decrease serotonin. This results in an increase of the sympathetic tone, an inhibitor of osteoblast proliferation (Ducy et al., 2000; Takeda et al., 2002). Insulin, produced in the β-islet cells of the pancreas, promotes osteoblast proliferation and differentiation (Fulzele et al., 2010). Peripheral serotonin produced by the enterochromaffin cells of the gut, binds to its receptor Htr1b on the osteoblast to inhibit proliferation (Yadav et al., 2008).

Ebf1 may not regulate bone mass accrual through its expression in the adipocyte

To begin addressing the question of how Ebf1 non-autonomously regulates osteoblast proliferation, we took cues from the phenotype of *Ebf1*$^{-/-}$ mice. These mice showed no significant changes in insulin levels, but did display a significant decrease in serum leptin, the inhibitor of bone mass accrual mentioned above (Fretz et al., 2010). We studied the role of Ebf1 in white adipocytes because *Ebf1*$^{-/-}$ mice are deficient in leptin, and also because white adipocytes are derived from the same mesenchymal progenitor as osteoblasts. Since MSCs give rise to all mesenchymal cell types, it is conceivable that additional transcription factors may promote differentiation along one lineage at the expense of differentiation along another. In particular, the possibility of reciprocal lineage choice between osteoblasts and adipocytes has

long been considered since the fat content of bones often increases when the number of osteoblasts and bone mass decreases (Rosen et al., 2009). Specifically, *PPARγ* favors adipocyte differentiation but inhibits osteoblast differentiation (Akune et al., 2004). We attempted to address the possibility that Ebf1 affects bone mass accrual through its expression in the white adipocytes. We performed expression analysis, cell culture experiments, and *in vivo* adipocyte-specific ablation of *Ebf1* using mice harboring a floxed allele of *Ebf1* that were crossed to *ap2-Cre* transgenic mice.

Previous experiments showed expression of *Ebf1* in white adipose tissue (Figure 2-1C). Using exonic qPCR primers normalized to genomic DNA, we confirmed that *Ebf1* was the most highly expressed of the *Ebf* family of transcription factors in primary white adipocytes (Figure 4-1A). Analysis of *Ebf1* expression in the embryonic fat depot at E14.5 and E16.5 also show that *Ebf1* transcript is present during development but very low levels. This low expression coincided with the strong one of *C/ebpα*, a determinant of white adipogenesis (Figure 4-1B).

We then examined whether decreasing *Ebf1* expression in a multipotential progenitor cell line, 10T1/2 cells, would promote expression of osteoblastic genes at the expense of adipocytic genes (Figure 4-2). Decreasing *Ebf1* expression by 94% resulted in a significant down-regulation of the major transcription factors responsible for determining adipocyte cell fate, i.e. *PPARγ, C/EBPα*, *C/EBPβ*, and *Prdm16*. In addition, expression of two adipokines *Adiponectin* and *Resistin* was similarly decreased. However, not all genes were equally affected. For instance, *C/EBPδ* and interestingly, *Leptin*, expression was unaffected by the decrease in *Ebf1* expression. Concurrently, *Ebf1* knockdown in 10T1/2 cells resulted in an almost four-fold upregulation of the main determinant of osteoblast differentiation, *Runx2,* and of several genes expressed in osteoblasts, such as *Col1a1, Alkaline phosphatase* (*Alpl*), and *Osteoprotegerin*

(Figure 4-2). In addition, ChIP analysis performed in 10T1/2 cells did not reveal Ebf1 binding to the promoters of any adipocyte-specific genes, only the *Alpl* gene which is expressed in osteoblasts (Figure 2-2B). Again, not all osteoblast-specific genes were affected by the down-regulation of *Ebf1*. Its knockdown did not affect expression of *Osterix, Atf4,* or *Osteocalcin*

Next, to further address this question we generated mice lacking *Ebf1* in adipocytes, crossing mice with a floxed allele of *Ebf1* with mice expressing *Cre recombinase* under the control of the *aP2* promoter. We confirmed that $Ebf1_{adp}^{-/-}$ mice lacked *Ebf1* in the adipose tissue only (Figure 4-3). $Ebf1_{adp}^{-/-}$ mice were born at the expected Mendelian ratio, indicating that complete *Ebf1* expression in cells of the adipocyte lineage is not necessary for embryonic development. At 1-month of age, $Ebf1_{adp}^{-/-}$ mice had normal body and epididymal fat pad weights (Figure 4-4A). Fat histological analysis further confirmed that adipocytes were present. Moreover, the relative distribution of adipocyte cell area was comparable in $Ebf1_{adp}^{-/-}$ and wild-type littermates (Fig. 4-4B). More surprisingly, and unlike what we observed in cell culture, expression of adipocyte and osteoblast marker genes was not affected by the absence of *Ebf1* in adipocytes (Fig. 4-4C). Altogether, these results indicate that, in the conditions of this experiment, *Ebf1* does not appear to noticeably affect cell differentiation *in vivo*.

We also analyzed the skeleton of these animals. Bone histomorphometric analysis performed at 1-month of age failed to show any change in bone mass, osteoblast number, and bone formation rate in $Ebf1_{adp}^{-/-}$ mice (Fig. 4-4D). These data indicate that removal of *Ebf1* from *aP2*-expressing adipocytes also does not overtly affect osteoblast differentiation.

We cannot exclude, however, the possibility that complete deletion of *Ebf1* or its removal in mesenchymal progenitor cells affects adipocyte and osteoblast differentiation. Unfortunately, no Cre driver line is yet available to address this question *in vivo*. That Ebf1 does not affect

Leptin expression suggests that it does not regulate bone mass accrual by modulating adipocyte production of leptin. The possibility that Ebf1 may be affecting bone mass accrual through its action in the certain cells of the brain, pancreas, gut, or other tissue(s), will need to be further tested.

The effect of *Ptpn2* deletion on insulin sensitivity decreases with age

The improved insulin sensitivity of $Ptpn2_{osb}^{-/-}$ mice was observed at 6 weeks of age. To determine if this insulin sensitivity was sustained as mice age, we tested the insulin sensitivity of older $Ptpn2_{osb}^{-/-}$ mice at 3 and 5 months of age. Compared to their wild-type littermates, insulin sensitivity of 3-month old $Ptpn2_{osb}^{-/-}$ mice was only moderately increased. At only two time points of the insulin tolerance test did the $Ptpn2_{osb}^{-/-}$ mice show significant improvement. In addition, the p-value accompanying the change in area under the curve was slightly above the point of significance (Figure 4-5A). Interestingly, the gonadal fat pad weight of 3-month old $Ptpn2_{osb}^{-/-}$ mice was significantly smaller compared to their wild-type littermates, a difference that was not observed in 6-week old mice (Figure 4-5B). By 5 months of age, any improvement of $Ptpn2_{osb}^{-/-}$ insulin sensitivity had disappeared (Figure 4-5C). This was noteworthy as the improved insulin sensitivity and glucose tolerance of *Esp* and $Esp_{osb}^{-/-}$ mice diminished by the time the mice reached 3 months of age.

That the phenotype of both osteocalcin gain-of-function models disappears with age raises additional questions. Does undercarboxylated osteocalcin lose its insulin-sensitizing capability with age-related insulin resistance? Do insulin-responsive tissues develop "osteocalcin resistance?"

It may also be possible that, with age, other PTPs begin to compensate for a lack of ESP or TC-PTP in osteoblasts. Increased PTP expression or activity may in fact contribute to the pathology of insulin resistance, as PTP expression has been shown to increase in an obesity state (Bence et al., 2006) (Loh et al., 2011). Further tests will need to be performed to determine if age and/or obesity increase the activity of PTPs in osteoblasts.

TC-PTP and ESP regulation of insulin signaling

TC-PTP is a negative regulator of insulin in the osteoblast, controlling whole-body insulin sensitivity and night-time energy expenditure through its regulation of osteocalcin activity. Its function parallels that of ESP action in the osteoblast. However, $Ptpn2_{osb}^{-/-}$ mice do not recapitulate the glucose intolerance and day-time increased energy expenditure observed in that of $Esp_{osb}^{-/-}$, suggesting that there exists a dissociation between osteocalcin action on insulin sensitivity and insulin secretion. In contrast to what has been previously observed, our results suggest that the threshold to affect insulin sensitivity is lower than that of insulin secretion (Ferron et al., 2008).

The difference among the various mutant mouse strains raises the question: do TC-PTP and ESP regulate insulin signaling in the osteoblast in a redundant, coordinated, or non-overlapping manner? To begin to address this question, we performed insulin tolerance tests on $Ptpn2_{osb}^{-/-}$;$Esp_{osb}^{-/-}$ mice to determine if TC-PTP and ESP function synergistically in the osteoblast to regulate whole-body insulin sensitivity. We tested these double mutant mice at 6 weeks of age, a time point in which the phenotype of $Ptpn2_{osb}^{-/-}$ display marked increased insulin sensitivity. However, we were unable to observe an increase in insulin sensitivity when mice lack both *Ptpn2* and *Esp* in the osteoblast, suggesting that TC-PTP and ESP may not be

coordinated in their regulation of insulin signaling (Figure 4-6). A caveat of this experiment, however, was the absence of a discernable change in insulin sensitivity of $Esp_{osb}^{-/-}$ mice at 6-weeks of age (Figure 4-6). These mice displayed an improvement in insulin sensitivity at 4-weeks of age, (Lee et al., 2007). Further analysis will be needed to address whether TC-PTP and ESP affect insulin sensitivity in a coordinated manner.

It may be useful to study the dynamics of ESP and TC-PTP regulation of the insulin receptor in the osteoblast. Previous studies in transformed mouse embryonic fibroblasts have demonstrated that TC-PTP is coordinated with PTP1B in the regulation of the insulin receptor. Regulation of the Y1162/Y1163 phosphorylation site differs between them in that TC-PTP is responsible for the sustained signal while PTP1B regulates signal intensity (Galic et al., 2003). As ESP and TC-PTP are localized to different regions of the cell, it is probable that their roles may also differ temporally. Further experiments will need to be performed to address the dynamics of ESP and TC-PTP.

Does TC-PTP regulation of osteocalcin bioactivity affect male fertility?

Osteocalcin increases the production of testosterone in males, and ESP has been shown to regulate the osteocalcin-testosterone axis. We measured testis weight in 3-month old $Ptpn2_{osb}^{-/-}$ males, the same age in which $Esp^{-/-}$ and $Ocn^{-/-}$ mice were analyzed for fertility (Figure 4-7) (Oury et al., 2011). Unlike what was observed in $Esp^{-/-}$ mice, however, we did not see a difference in testis weight. The possibility that TC-PTP may regulate the osteocalcin-testosterone axis remains to be more fully explored.

Are there other TC-PTP substrates in the osteoblast?

We have observed that the IGF-1R receptor is an *in vitro* substrate of PTP1B, TC-PTP, and ESP (Figure 4-7). A GST-pull down performed ROS cells reveals that PTP1B, TC-PTP, ESP, but not PTPRJ, bind insulin-like growth factor type 1 receptor (IGF-1R) (Figure 4-8). Interestingly, TC-PTP and ESP wild-type mutants can bind to IGF-1R independently of their catalytic domain (Figure 4-8). IGF-1R has previously been shown to be necessary in osteoblasts for growth hormone (GH)-dependent proliferation (Fulzele et al., 2010) (DiGirolamo et al., 2007). However, in our studies, we did not observe an increase in osteoblast number in *Ptpn2*$^{-/-}$ mice (Figure 3-5G,F). The possibility remains that TC-PTP has additional substrates in the osteoblast.

Does TC-PTP regulate hematopoiesis through its expression in the osteoblast?

TC-PTP has been shown to be an important regulator of hematopoiesis and the immune response. Through cell-autonomous regulation of the JAK/STAT pathway and CSF-1 signaling, TC-PTP regulates cytokine production and mononuclear infiltrates (ten Hoeve et al., 2002) (Simoncic et al., 2002). The demonstration that TC-PTP regulates osteoclast maturation through its expression in the osteoblast suggests that development of other hematopoietic cells may also be affected by TC-PTP function in the osteoblast. In fact, TC-PTP has been shown to regulate B-cell development through its expression in bone marrow stromal cells that express interferon-γ (Bourdeau et al., 2007).

What is the role of TC-PTP in human osteoblasts?

SNPs in the human *PTPN2* gene have been associated with the development of type 1 diabetes (Smyth et al., 2008) (Espino-Paisan et al., 2011). Since the skeleton is an important contributor to whole body glucose homeostasis, it is possible that TC-PTP plays an important role in human osteoblasts to regulate whole-body insulin sensitivity. To gain a better understanding of its role in humans, TC-PTP function should be studied in the context of a human osteoblast cell, similar to what has been previously done with the study of human PTP1B (Ferron et al., 2010).

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Figures

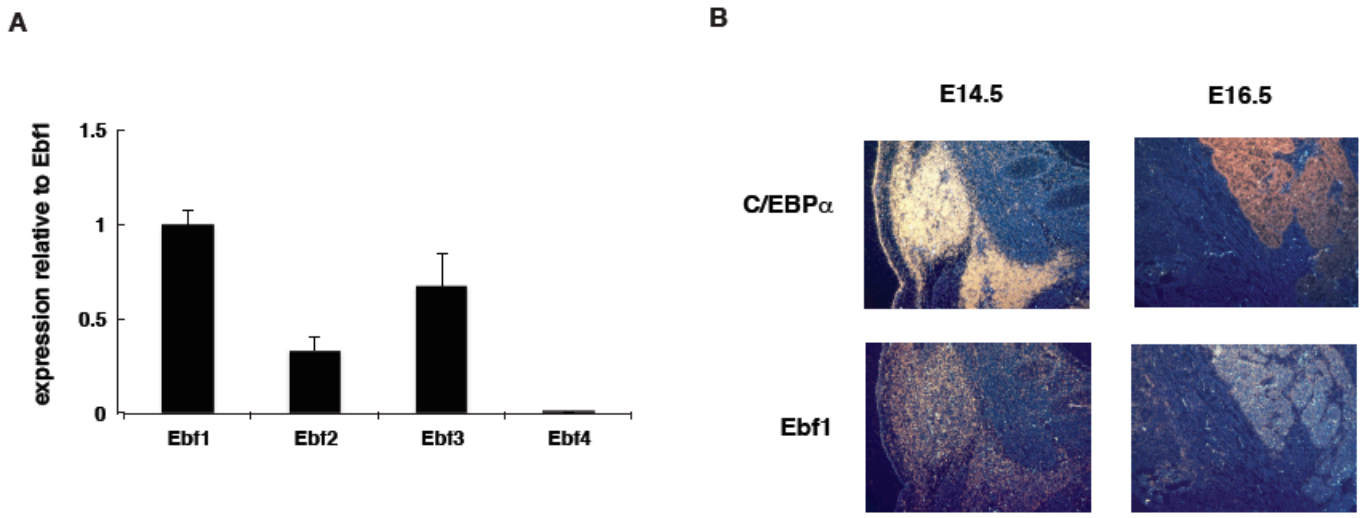

Figure 4-1. Ebf1 expression analysis in adipocytes. (**A**) Normalized expression of *Ebf1, 2, 3,* and *4* by qPCR in primary adipocytes. (**B**) Expression pattern analysis of *Ebf1* by (radioactive *in situ* hybridization of adjacent sections featuring the embryonic (*C/EBPα*) fat depot in mouse embryos.

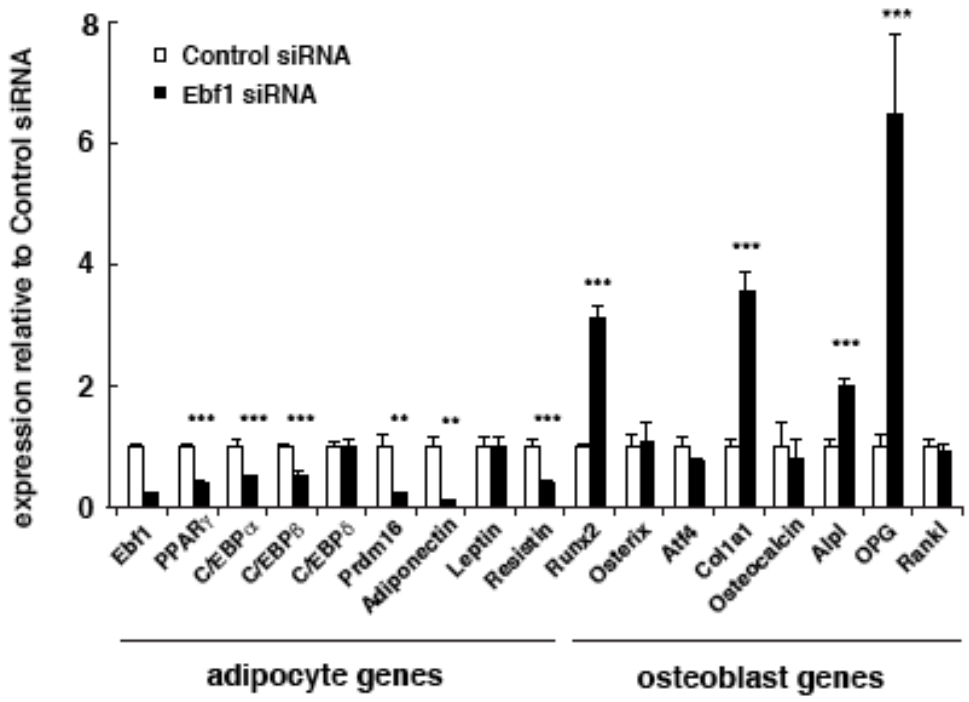

Figure 4-2. Ebf1 siRNA knockdown. 10T1/2 cells cultured for 2 days in adipocyte differentiation medium,

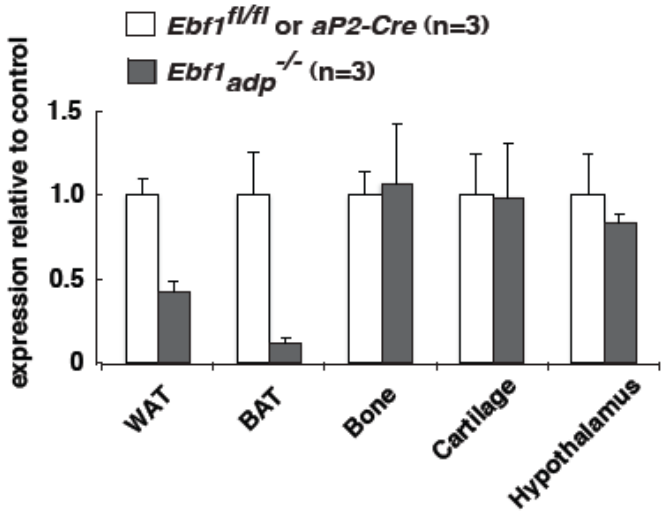

Figure 4-3. Deletion of the *Ebf1* allele in indicated tissues.

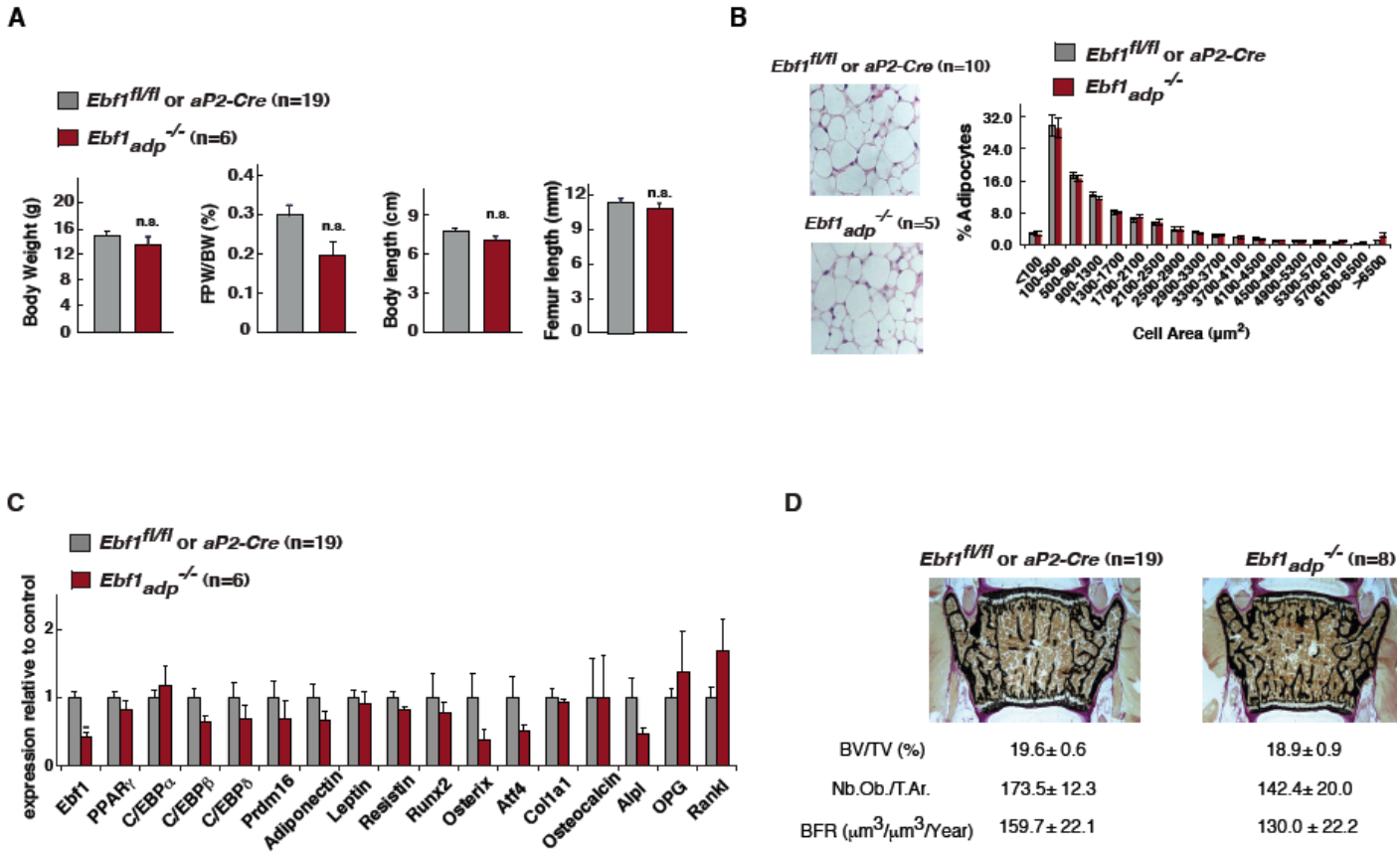

Figure 4-4. Phenotype analysis of *Ebf1*$_{adp}^{-/-}$. (A) Body weight, percent gonadal fat (FPW/BW), body length, and femur length (B) distribution of adipocyte cell size (C) qPCR analysis of white adipose tissue, and (D) bone histomorphetric analysis of *Ebf1*$_{adp}^{-/-}$ mice.

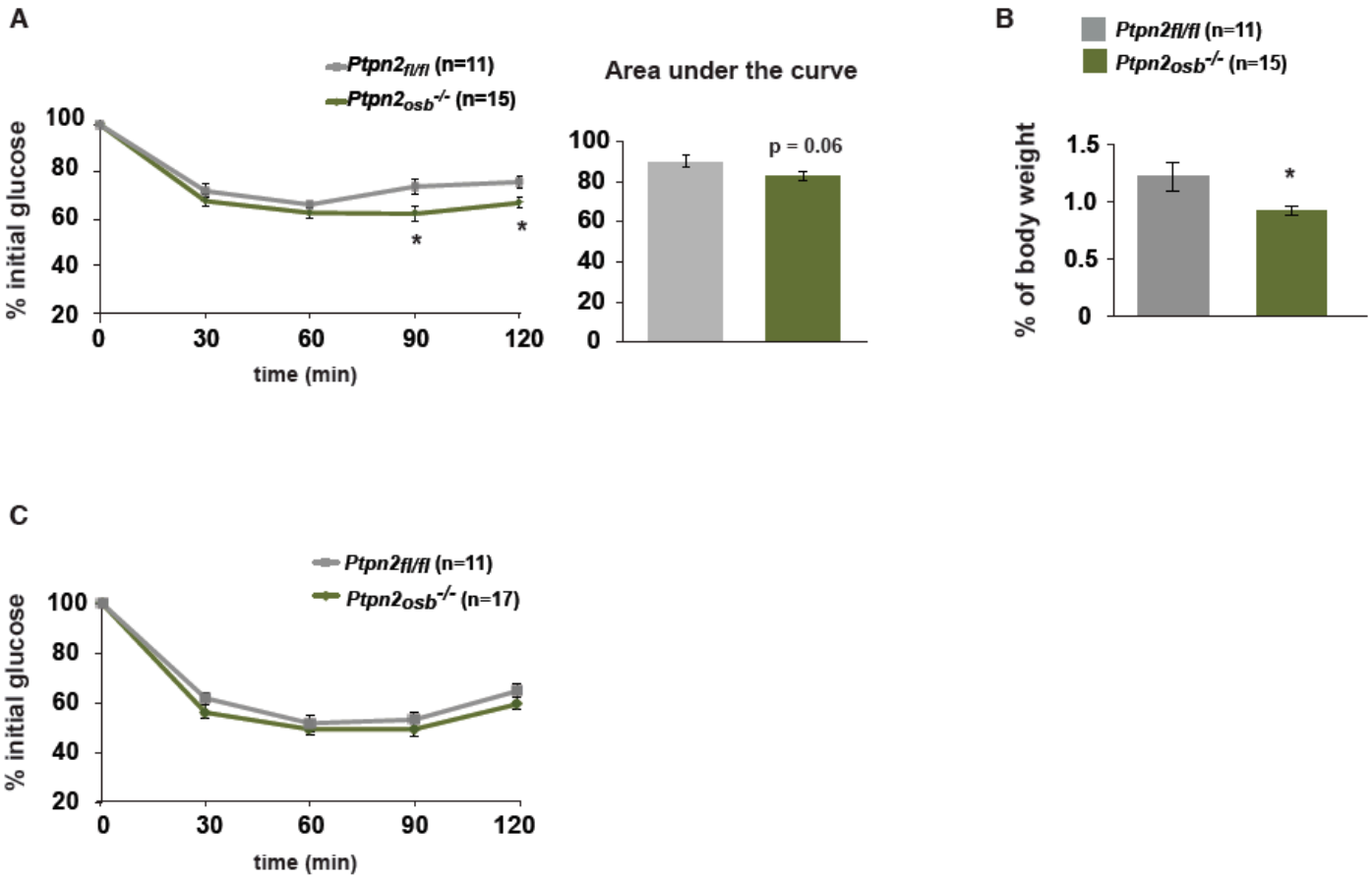

Figure 4-5. Insulin sensitivity of aged *Ptpn2*$_{osb}^{-/-}$ mice. (**A**) Insulin tolerance test on 3-month old *Ptpn2*$_{osb}^{-/-}$ male mice with the area under the curve. (**B**) Fat pad weight of 3-month old *Ptpn2*$_{osb}^{-/-}$ mice. (**C**) Insulin tolerance test on 5-month old *Ptpn2*$_{osb}^{-/-}$ male mice

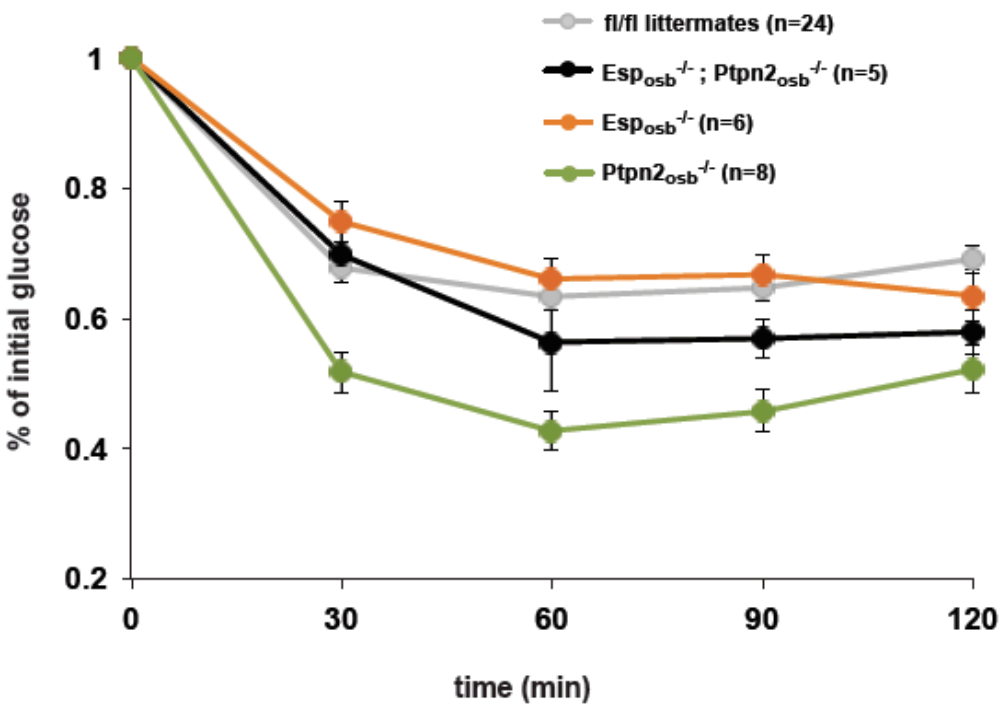

Figure 4-6. Insulin sensitivity of *Ptpn2$_{osb}^{-/-}$;Esp$_{osb}^{-/-}$* double knockout mice. Insulin tolerance test of 6 week old male mice.

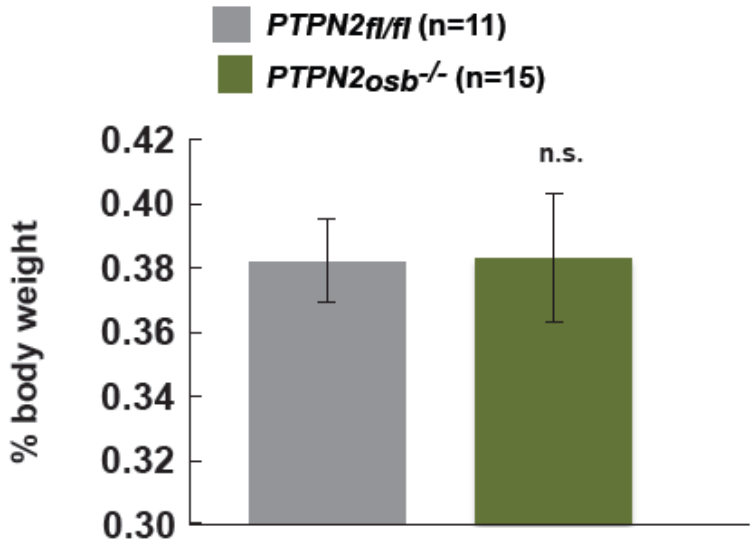

Figure 4-7. Testis weight of 3 month old *Ptpn2*$_{osb}^{-/-}$ mice.

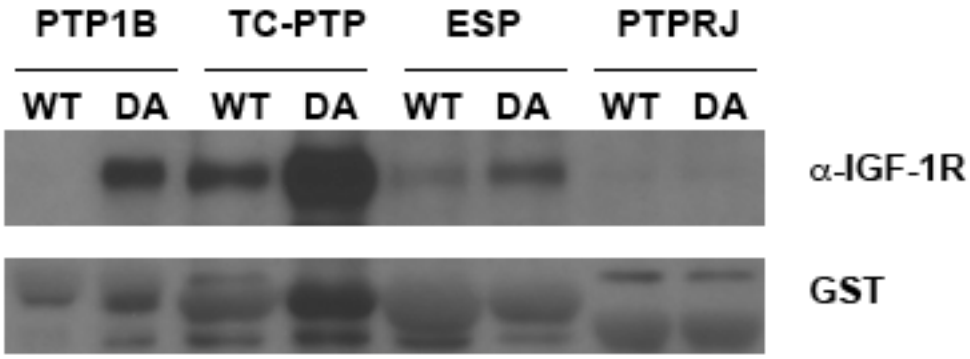

Figure 4-8. In vitro substrate trapping. Extracts from pervanadate-treated ROS17/2.8 cells were pulled down using GST only or DA mutants of PTP-GST fusion proteins. Insulin-like growth factor 1 receptor (IGF-1R) was detected by Western blotting.

Chapter Five: Perspective

Mice lacking the transcription factor *Ebf1* in all cells have high bone mass due to an increase in bone formation (Hesslein et al., 2009). To test this whether Ebf1 inhibits osteoblast function, we analyzed *Ebf1* function in primary osteoblasts and *in vivo* through osteoblast-specific inactivation in in the mouse, and demonstrated that deletion of *Ebf1* in early osteoblast cells does not increase osteoblast differentiation or function.

The finding that the Ebf1 does not regulate bone mass through its expression in the osteoblast demonstrates that this transcription factor regulates bone formation through its expression in another cell type. One distinct possibility is that Ebf1 regulates bone mass through its expression in the dorsal root ganglia and spinal cord, areas that contain the axons of primary afferent neurons and that we have identified as expressing Ebf1 at high levels (unpublished data). That the central nervous system plays an important role in the regulation of bone remodeling has been previously established with the discovery that leptin inhibits bone formation via a hypothalamic relay and mediation of the sympathetic tone (Ducy et al., 2000) (Yadav et al., 2009). The sympathetic signal is thus sensed by osteoblasts, which express functional β2-adrenergic receptors on their cell surface (Takeda et al., 2002). The discovery of a brain-bone link suggests that bone remodeling is tightly controlled by the nervous system, and this relationship may indeed extend beyond that of hormonal control, suggested by the presence of afferent neurons that physically connect the bone to the central nervous system. Yet the mechanisms through which sensory neurons regulate bone formation and/or bone resorption are unidentified (Chenu, 2004) (Elefteriou, 2008). I propose here that Ebf1 regulation of bone mass accrual occurs through its expression and function in afferent neurons, and that Ebf1 acts as a repressor of sensory neuron activation.

The clinical observation that pain is experienced in the bones has long indicated that the skeleton is innervated with sensory neurons. The activity of sensory neurons in the bone may be important for two main reasons. First, the nervous system must be able to sense skeletal fracture in order to stimulate endochondral ossification and bone remodeling at the site of trauma. Secondly, the skeleton must also sense changes in mechanical load in order for it to adapt to changes in weight and external impact. This functional adaptation is supported by observations in human studies that describe strong correlations between high-impact exercise and increased osteogenic response resulting in high bone mass; in contrast, inactivity is associated with diminished bone mass (Bassey and Ramsdale, 1994) (Courteix et al., 1998) (Robinson et al., 1995) (Taaffe et al., 1997) (Chenu, 2004). It has since been demonstrated experimentally in rodents that mechanical stress and loading are important factors in the determination of bone volume in both cortical and trabecular regions (Mosley and Lanyon, 1998; Sugiyama et al., 2010) (Sugiyama et al., 2010) (Carter et al., 1987). These results are supported by a computational simulation model that confirms trabecular bone architecture is highly adaptive to mechanical load (Huiskes et al., 2000).

The most recognized site of nociception in the skeleton is at the periosteum, the connective membrane that surrounds the outer cortex of mineralized bone, excluding the joints of long bone. The periosteum is composed of two layers: its outer layer consists of fibroblasts and collagen, while the inner bone collar is made up of mainly osteoprogenitor cells that are recruited to expand the cortical thickness of bone and repair fractures. Nerve endings, including those of the peptidergic calcitonin-gene related peptide immunoreactive (CGRP-ir), as well as myelinated sensory neurons, are found within the inner periosteum region in close proximity to osteoblast progenitors and within the Haversian and Volkman's canals of the compact bone (Hara-Irie et

al., 1996) (Mach et al., 2002). Interestingly, CGRP-ir fibers surrounding the bone collar undergo rapid proliferation and sprouting upon bone fracture, demonstrating that these neurons are responsive to changes in bone integrity and thus, may have an important role in fracture repair (Hukkanen et al., 1993).

It has been long thought that the periosteum is the only area of nociception in the skeleton. However, studies have identified CGRP-ir fibers, myelinated sensory fibers, and sympathetic nerve fiber endings in the regions of mineralized bone and bone marrow, and at a higher density than that of the periosteum when calculated per volume (Kuntz and Richins, 1945; Serre et al., 1999) (Mach et al., 2002). The direct contact of nerve fibers with osteoblasts in these areas suggests a regulatory role for sensory input in the regulation of bone turnover and remodeling in not only cortical bone, but also in the trabecular areas that are responsive to mechanical load and stress.

The cell bodies of primary afferent neurons, whose synaptic terminals reach into the spinal cord for signal transduction to the brain, are contained in the dorsal root ganglia. Ebf1 expression is high in both the dorsal root ganglia and spinal cord, suggesting that this transcription factor may affect the ability of sensory neurons to transmit signals to the central nervous system. That rapid and ubiquitous osteoporosis develops following spinal cord injury demonstrates that intact spinal cord function is critical for the maintenance of bone mass. This observation, combined with analysis showing high bone mass in *Ebf1*$^{-/-}$ mice, indicates that if Ebf1 were to function in sensory neurons, it would be a repressor of afferent neuron activation.

In support of this hypothesis is the observation that Ebf1 can function as both an activator and repressor. In B cells, Ebf1-repressed targets display a loss of activating chromatin marks and gain of H3K27 trimethylation (Treiber et al., 2010). ChIP-seq can be performed in isolated

dorsal root ganglia and spinal cord tissue to identify these Ebf1 target genes, similar to what has been previously done in B cells (Treiber et al., 2010). As a "pioneer factor," Ebf1 may be involved in early specification sensory neuronal fate or the expression of neurotrophic factors that are important for sensory neuronal survival.

Furthermore, to test the hypothesis that Ebf1 regulates afferent neuron activation *in vivo*, *Ebf1* should be genetically ablated specifically in primary afferent neurons. $Ebf1^{fl/fl}$ mice should be crossed to mice expressing Cre Recombinase under the control of a promoter expressed specifically in primary afferent neurons – for example: *Prph-Cre* or *Pvalb-Cre*. If the above hypothesis is correct, $Ebf1_{DRG}^{-/-}$ mice will display an increase in bone mass as a result of increase bone formation and decreased osteoclast function, similar to what was observed in $Ebf1^{-/-}$ mice (Hesslein et al., 2009).

In addition to high bone mass and a lack of B cells (Hesslein et al., 2009; Lin and Grosschedl, 1995), $Ebf1^{-/-}$ mice display runted growth, decreased body adipose, increased marrow adipose, and abnormal glucose metabolism (Fretz et al., 2010), conditions that may all contribute to the low survival rate of these knockout mice on the C57Bl/6 genetic background. That $Ebf1^{-/-}$ mice have a multitude of abnormalities further supports the hypothesis that Ebf1 functions in the central nervous system, and suggests that $Ebf1^{-/-}$ mice are a gain-of-function of sensory neuron activation. It would be interesting to see if Ebf1 deletion in the dorsal root ganglia neurons recapitulates any of the abnormalities observed in the total knockout.

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